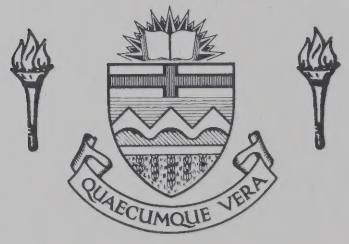

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THE UNIVERSITY OF ALBERTA

GENETIC MARKERS IN THE CHICKEN

by

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SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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ABSTRACT

Eleven forms of molecular variation reported for the chicken was re-examined. Variation was demonstrated for hemoglobin, alkaline phosphatase, and "Hi" agglutinogen. Three hemoglobin phenotypes were found, confirming an earlier report. The phenotypes appear to be determined by a pair of co-dominant alleles. Three phenotypes of alkaline phosphatase were found, in agreement with one report and in disagreement with another. Two phenotypes were found for the "Hi" system, in agreement with earlier reports.

The "Hi" system is based on the agglutination of erythrocytes from adult females by lectins from Pisum arvense. The conditions of this agglutination and the characteristics of the lectin were examined in some detail. Agglutination was insensitive to temperature and pH and to pre-treatment of the erythrocytes with neuraminidase and was inhibited by D-mannose. Agglutination was not inhibited by several other sugars reported to be inhibitory. Fluorescein-labelled lectin labelled agglutinable erythrocytes establishing that the lectin actually binds to the erythrocyte. The agglutinating activity was found in two proteins separated by isoelectric focusing. These two proteins migrated differently in thin layer SEPHADEX gel. The molecular weights estimated from these migrations are low for lectins and may represent a degree of binding to SEPHADEX.

ACKNOWLEDGMENTS

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Introduction

The chicken was one of the first animals in which Mendelian inheritance was demonstrated. This animal has been the subject of genetic research for economic as well as scientific reasons. As a consequence, a number of morphological and molecular markers have been discovered. Before discussing the markers, it is desirable to review the linkage map of the chicken.

I. The Linkage Map of the Chicken

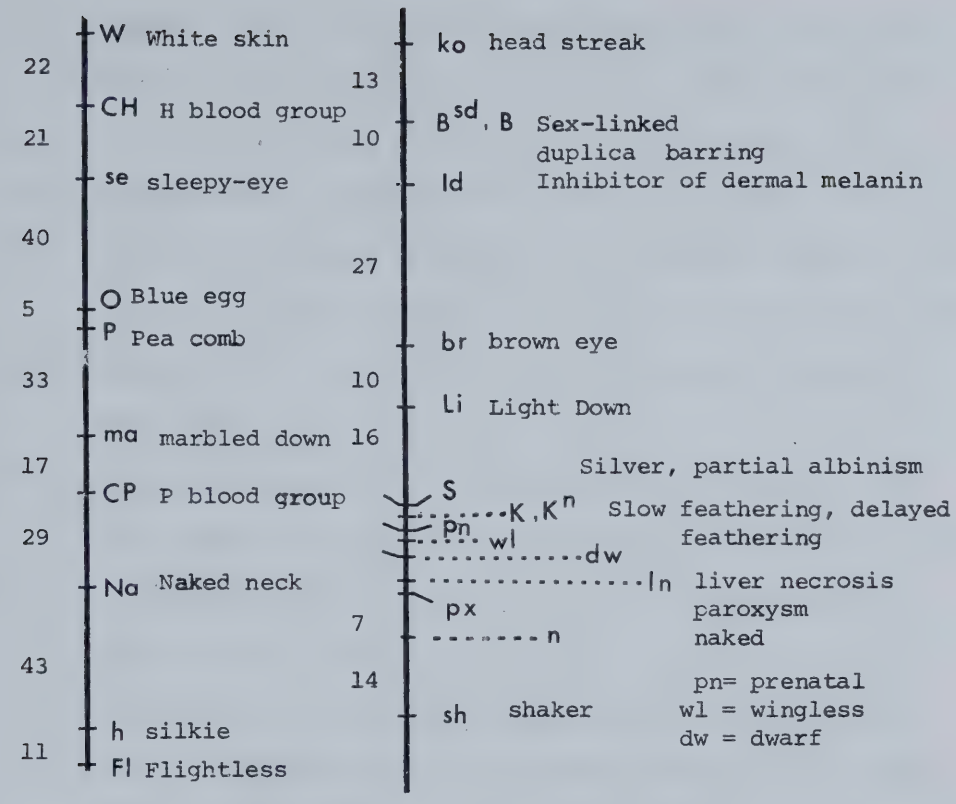
The first linkage map of the chicken was published by Hutt in 1936. Since then, a number of additions to the linkage groups have been made, despite slow progress in the mapping of morphological and molecular markers. Basically, the linkage map of the chicken consists of 14 loci on the sex chromosome (Z) and 16 autosomal loci in 4 or 5 linkage groups as shown on the next page. It may be of interest to note that most traits on the sex chromosome (Z) are recessives, while most of the others are dominants.

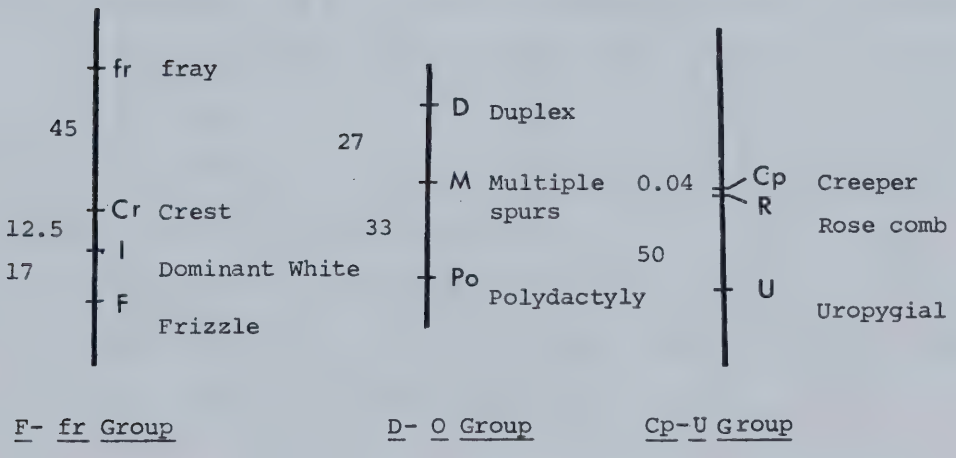
A number of mutants are known to exhibit sex-linked inheritance but have not been mapped:

(i) sex-linked lethal (xl) - a condition in which the pullets fall into a semi-comatose state in the mornings and die usually within a few hours (Hutt, F. B., 1960).

(ii) Jittery (j) - expressed as a neurological disorder which leads to death soon after hatching (Hutt, F. B., 1960).

(iii) gasper (g) - a recessive sex-linked mutant; expressed as a respiratory defect, causing bronchial gasping (Price, D. J., et al., 1966).





Linkage Map of the Chicken (After Etches, R. J., and Hawes, R. O., 1973).

(iv) Coloboma (co) - a sex-linked mutant which expresses a primary effect on beak and eyes and both sets of limbs; greatest reduction of the wings (Abbott, U. K., et al., 1967, 1970).

(v) dwarfing (dw^B) - a mutant which maps in the region of S and K has been reported by Jaap (1969, 1971) who also suggested that this gene may be an allele of the recessive dwarfing gene (dw).

(vi) baldness (ba) - a gene shown to be a sex-linked recessive by Somes (1970).

(vii) chondrodystrophy (chz) - a lethal, sex-linked recessive gene which causes defects in cartilage formation (Mann. G.E., 1963).

(viii) Z histoantigen (z) - an histoantigen locus hypothesized by Bacon and Craig (1967).

(ix) ladykiller (lk) - a gene whose phenotypic expression is very similar to the prenatal (pn) gene was identified by Sheridan (1964). It is possible that the genes pn and lk are identical, but unfortunately classical tests for allelism cannot be carried out as only carrier males are available for breeding.

(x) diplopodia-4 (dp^4) - a sex-linked recessive lethal to the hemizygous females during the terminal stages of incubation (Abbott, U. K., 1967).

Similarly, a number of traits of linkage group III has been recently assigned to chromosome 1, but not mapped:

(i) perosis (pe) - an autosomal recessive gene responsible for the congenital condition known as "slipped tendon"; it is believed to be linked to the sleepy eye locus.

(ii) feather achromatosis (mi) - an autosomal recessive gene causing reduction in melanin deposition; possibly linked to the pea comb gene (Washburn, K. W., and Smyth, J. R., 1967).

(iii) tardy feathering (t, t^s) - a locus determining a bare back condition; recessive to normal allele (T) for normal feathering (Hutt, F. B., 1949).

The various assignments of blood group and histocompatibility loci to the linkage groups are tentative and presently rather uncertain in some cases. From a study of the linkage relationships between 10 blood group loci and the loci of 7 morphological traits, Briles et al. (1967) assigned the P blood group locus to chromosome 1 based on its linkage with naked neck of 29.1 percent. In addition, the authors also added the J blood group locus to this linkage group, based on 40.7 percent linkage with pea comb and H blood group locus with white skin, based on a linkage of 22.1 percent. Lastly, the B locus coding for the major histocompatibility antigens has been assigned to the D-Po linkage group by Briles et al. (1967), based on a suggested linkage of 46.1 crossover units between polydactyly (Po) and the B histocompatibility locus. However, subsequent work revealed that the suggested linkage between Po and B is rather dubious. Since no evidence exists to suggest that the B histocompatibility locus belongs to the other linkage groups (Briles, C. et al., 1950), it is quite likely that the B locus may reside in one of the many microchromosomes (Pazderka, F. et al., 1975).

It should become apparent that progress in assignment of blood group and histocompatibility loci to other linkage groups or microchromosomes will ultimately depend on the number of morphological and molecular markers that will become available to us. Consequently, a

search for new molecular markers and confirmation of reported markers is fully justified for the following reasons. As one accumulates a number of molecular and immunological markers, one may discover that some of the markers might be associated with important functional differences. If optimism permits, one might be able to demonstrate that a particular biochemical variant (marker) serves a specific function *in vivo*. Even if one fails to find functional associations, establishment of an array of molecular markers will at least provide useful probes for genetic and physiological research.

When one embarks on a search for molecular markers, one is faced with the problem of what to use as a source of biochemical variants. Strategically, one should use material which is easily and repeatedly accessible and removal of which does not impair the animals' viability or health. This eliminates tissue samples (obtained via biopsies). Blood (especially serum or plasma) as a source of blood-borne markers seems to be the soundest choice. Promising markers present in the blood of the chicken need not come from blood cells. Some are synthesized by the liver or intestinal epithelium. The cell sources for the markers to be discussed later are as follows:

Marker:	Main Cell Source:
transferrin	hepatic cells
albumin	hepatic cells
prealbumin	hepatic cells
catalase	erythrocytes
hemoglobin	erythrocytes
riboflavin binding-protein	hepatic cells
Hi agglutinogen	erythrocytes
amylase	hepatic and pancreatic cells
alkaline phosphatase	osteoblasts and intestinal epithelial cells
leucine aminopeptidase	intestinal epithelial cells

II. A Survey of Genetic Markers in the Chicken

The purpose of the sections to follow is to attempt to briefly outline some of the biochemical variants or genetic markers in the chicken.

Transferrin

Transferrin, an iron binding protein present in chicken serum is polymorphic. In 1962, using starch gel electrophoresis Ogden *et al.* reported the presence of three transferrin isozymes in chicken sera. They proposed that the isozymes are the products of two alleles at a single autosomal locus. The genotype designations used by the authors were Tf^a and Tf^b for the alleles, and Tf-a, Tf-ab and Tf-b for the three observed phenotypes.

Contrary to Ogden's findings, Vyshinsky and Muraviev (1968) reported finding five transferrin phenotypes out a proposed list of six expected phenotypes using disc gel acrylamide electrophoresis. Based on the variations observed, they suggested that chicken transferrins are controlled by three alleles at one locus with six corresponding phenotypes and not by two alleles with three corresponding phenotypes as proposed by Ogden et al. (1962). However, the results of blood analysis of offspring from matings of transferrin typed parents are inconclusive due to small sample sizes and lack of appropriate mating types. Confirmation of Ogden's findings was provided by Stratil (1968) six years later. Using starch gel electrophoresis, autoradiography, and appropriate mating studies, Stratil demonstrated that differences in transferrins are determined by three alleles, namely Tf^a, Tf^b, and Tf^c.

Attempts to confirm the mentioned findings were unsuccessful. Using disc gel acrylamide electrophoresis, plasma samples from out stock of chickens were tested for the presence of transferrin variants employing the iron specific staining technique of Mueller et al. (1962). No visible bands could be detected after staining. Perhaps more Fe^{+++} ions in the form of ferric ammonium sulphate could have been added to the plasma samples prior to electrophoresis or possibly the level of transferrin in the samples was too low to be detected by the above mentioned detection technique.

Albumin

Albumin, a major protein in chicken serum, is also polymorphic. Using starch gel electrophoresis, McIndoe (1962) observed two genetic variants of albumin for which he gave the designations F for the fast migrating variant and S for the slower migrating variant. McIndoe reported that using the alkaline buffer system heterogeneity of the serum albumin could not be demonstrated. In his electrophoretic separations, the author used a buffer system with a pH of 5.4. In the same study, the results of matings involving SF X SF, S X F, SF X S and S X S were shown by McIndoe to be consistent with a simple Mendelian inheritance of the two variant albumins.

A preliminary experiment involving 35 plasma samples from chickens of a wide range of B genotypes revealed that the two variants of albumin do indeed exist. The plasma samples were subjected to disc gel acrylamide electrophoresis using a Tris-glycine buffer of pH 8.3. Following completion of the electrophoretic run, the gel columns were stained with Buffalo Black (0.1% in 7% acetic acid) to reveal the albumin bands. The stained gel columns were scanned densitometrically and the migration distances from the origin were measured for all the albumin bands from the obtained densitometric records.

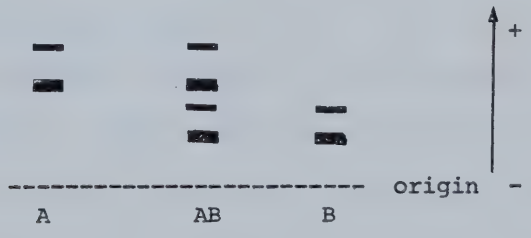
The migration distances were divisible generally into two groups: a fast group with a mean migration distance of 13.7 cm (as measured from the scan records) and a slow group with a mean migration distance of 12.2 cm. Contrary to what was expected, the number of observed fast albumin variants (n=28) was greater than the number of slow migrating albumin variants (n=7). The results of the Students' t-test showed that the mean migration distances between the fast and slow groups are

statistically significant ($P < 0.01$).

It must be noted that the difference in migratory distances between the fast albumin and slow albumin variant is not dramatic; there were some acrylamide gel columns in which one could not readily classify or type the albumin band without a known fast or slow albumin variant on hand for comparison.

Prealbumin

Variants of prealbumin are believed to exist in the serum of chickens. Employing starch gel electrophoresis, Stratil (1970) observed three different variants of prealbumin. Two of the variants were represented by one stronger band and one weaker, more anodic band. The third variant consisted of four bands located in the positions of the zones of the above mentioned variants. The reported variants may be shown diagramatically as follows:



The results of mating studies seem to suggest that the prealbumin phenotypes are genetically determined at one autosomal locus by two codominant alleles, Pa^A and Pa^B.

It appears that prealbumin may be a good potential genetic marker. However, preliminary experiments revealed that the prealbumin bands were too faint to permit undubious typing. Perhaps larger sample volumes (greater than 20 microliters) might make the prealbumin bands more

intense so as to allow positive identification.

Catalase

While studying iron metabolism in chickens infested with erythroblastosis, Bather et al. (1963) found that the blood catalase levels of East Lansing Line 15 White Leghorns fall into three sharply defined groups:

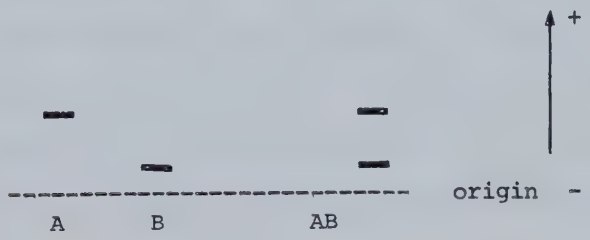
(i) 100-500 units of catalase activity

(ii) 700-1200 units of catalase activity

(iii) over 1300 units of catalase activity

These workers proposed that the catalase levels are the results of three genotypes, cc with little catalase activity, Cc and CC with higher catalase activities.

Confirmation of the reported findings was obtained about nine years later. While attempting to correlate the frequency of blood group antigens with blood catalase levels, Ermencova and Shabalina (1972) discovered that the blood catalase level of six breeds of chickens fall into three classes, namely low, intermediate, and high. In the same year, Shabalina (1972) employing starch gel electrophoresis and the catalase detection technique of Tudhope (1965) managed to demonstrate the existence of electrophoretic variants of catalase in the chicken. The three catalase isozymes observed by Shabalina are diagramatically represented below:



In the same study, the distribution of the genotypes CtAA, CtBB, and CtAB in various fowl populations was determined, but unfortunately no segregation experiments were conducted to prove that the observed catalase isozymes are under simple Mendelian control.

A preliminary experiment employing disc gel electrophoresis and the catalase detection method of Baumgarten (1963) gave rather dubious results. Twelve hemolysate samples from chickens of a wide range of B genotypes were subjected to disc acrylamide electrophoresis for 1½ hour. After completion of the electrophoretic run, the gel columns were treated with o-dianisidine according to the catalase staining method of Baumgarten (1963) to locate the sites of catalase activity. Each hemolysate sample gave rise to a single faint band approximately 2 mm away from the origin. It seems that the slower migrating catalase variant was detected, but it is not safe to make such a claim until the intensity of the catalase bands is improved.

Riboflavin-Binding Protein

Certain families of Single Comb White Leghorns are deficient in a riboflavin-binding protein. In fact, the deficiency is so great that unless the embryos of these chickens are given supplements of riboflavin, they will all die between the 10th and 14th days of incubation (Maw, A. J. G. 1954).

Maw (1954) was the first to suggest that a recessive gene is responsible for the inability of homozygous (rd/rd) birds to lay eggs with sufficient riboflavin to enable the embryo to survive. Twelve years later, Cowan et al. (1966) proposed that the recessive gene expresses itself by altering the renal reabsorptive mechanism for free

riboflavin. As a result of this alteration, the absorbed riboflavin is rapidly excreted via the urine after absorption and hence it does not accumulate in the blood. With a rather limited supply of riboflavin in the blood, transfer to the egg is restricted.

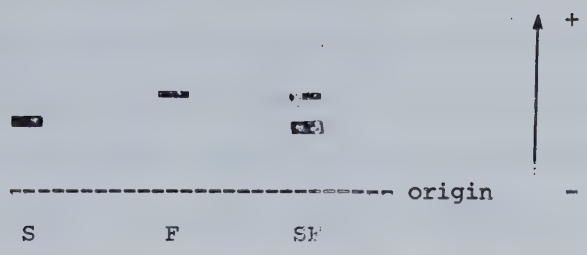
Studies of riboflavin binding capacities of serum, albumin and yolk using the technique of equilibrium dialysis revealed that one could differentiate RdRd from rdrd (recessive) chickens on the basis of the riboflavin binding capacities of their sera. The reported riboflavin binding capacities of serum are as follows: (Winter, W. P., et al. 1967).

Genotype:	Riboflavin Binding Capacity (in microgram/ml)
RdRd	1.18
rdrd	0.12

Presumably, serum from a heterozygous bird (Rdrd) will have a riboflavin capacity mid-way between the previously mentioned values. If this should prove to be the case, then one should be able to type any chicken purely on the basis of riboflavin binding capacity of its serum.

Amylase

Using disc acrylamide electrophoresis, Heller and Kulka (1968) discovered two isozymes of amylase in homogenates of chick pancreas. Studies on pancreatic homogenates of individual chicks revealed three phenotypes which may be shown diagramatically as follows:

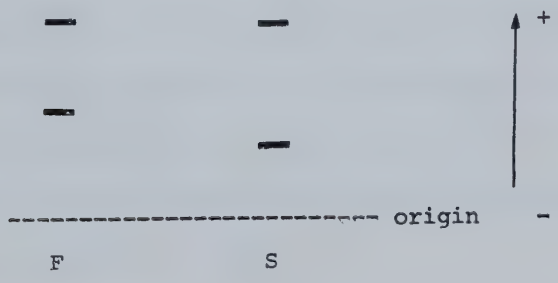


The authors suggested that the slow and fast amylase isozymes are the products of two allelic genes. In view of these findings, it is quite possible that amylase isozymes might also be present in the serum.

Preliminary work indicated that this possibility indeed exists. However, the amylase bands were very weak possibly due to low levels of amylase in serum. It appears that once a more appropriate amylase detection technique is attained, amylase may become another added genetic marker.

Leucine Aminopeptidase

According to Law (1967), two phenotypes of leucine aminopeptidase exist in the plasma of chickens. The fast migrating form consists of two bands and the slow form consists of two bands with one band migrating close to the origin. The observed phenotypes may be illustrated as follows:



Law also reported the observation that the two electrophoretic variants of leucine aminopeptidase show a direct association with the two genetically controlled forms of alkaline phosphatase. In other words, all plasma samples showing the fast form of leucine aminopeptidase also exhibited the fast form of alkaline phosphatase (Ap^2). The author postulated that the two forms of the enzymes may be due to the presence or absence of a single gene (perhaps coding for a sialyl transferase) controlling the attachment of sialic acid to alkaline phosphatase and leucine aminopeptidase.

The above findings were later confirmed by Csuka and Petrovsky (1972). These authors also pointed out that only one slight band appeared after staining unless a five-fold increase of the substrate concentration was used.

A preliminary experiment in which 48 plasma samples from chickens of a wide range of B genotypes revealed only a single migrating band with a mean mobility of 47.4%. Simply on the basis of this finding, one could not determine whether the observed band was the fast leucine aminopeptidase isozyme or the slow migrating form. Perhaps use of a higher substrate concentration as pointed out by Csuka and Petrovsky (1972) might resolve this problem.

The three other remaining genetic markers, alkaline phosphatase, hemoglobin, and "Hi" agglutinogen, not discussed in this general survey of available markers, were investigated in greater detail in the present study. Investigations and attempts at confirmation of these markers have turned up significant, novel findings which are presented in the three chapters to follow.

Chapter 1

"Hi" Agglutinogen

Introduction

The agglutination of erythrocytes by plant extracts has been known since the turn of the century (Makela, O., 1957; Bird, G. W. G., 1959; Boyd, W. C., 1963; Sharon, N. and Lis, H., 1972). The agglutinations are due to proteins which are most readily obtained from the seeds of legumes, but are present in roots, leaves, and bark (Bird, G. W. G., 1959; Boyd, W. C., 1963; Makela, O., 1957; Toms, G. C. and Western, A., 1971). Extracts of snails, horseshoe crabs, and some other invertebrates and lower vertebrates show similar activities (Pardoe, G. I. and Uhlenbruck, G., 1970). The terms "phytoagglutinins" and "phytohemagglutinins" introduced to indicate the origin of the first active extracts have been superseded by "lectins". A lectin is a naturally occurring non-enzymatic protein which binds to erythrocyte surfaces. This definition is subject to qualification as our knowledge of protein interactions grows.

The binding of lectins is not limited to erythrocytes. Some lectins bind to lymphocytes and stimulate them to proliferation (Nowell, 1960). Some combine with blood group substances obtained from cells other than erythrocytes. Some discriminate between cultures of normal and transformed cells (Aub, J. C. et al., 1963; Burger, M. M., and Goldberg, A. R., 1967; Inbar, M. and Sachs, L., 1969; Sela, B. A. et al., 1970). These reactions are initiated by combination of the lectin with a monosaccharide constituent of an oligosaccharide side-chain or a longer and more complex saccharide side-chain of a protein. The gross effects on cells are attributed to alterations of the cell surface secondary to the initial binding. It is usually presumed that a lectin has more than one binding site per lectin molecule and the

receptor protein has more than one binding site per receptor molecule (Sharon, N. and Lis, H., 1972). The reaction is a mixed polymerization tantamount to the reaction of antibody and antigen. The specificity of these reactions is thought to be entirely due to the initial binding to monosaccharide. The best evidence for this is the selective prevention of lectin-induced effects by the presence of an excess of a particular monosaccharide (Makela, O., 1957; Goldstein, I. et al., 1965). Different lectins are inhibited by different monosaccharides. This selectivity permits us to deduce the identity of the structure which combines with the lectin. To date this is always a monosaccharide. A reaction initiated by a lectin has, therefore, the potential of serving as a tool for the detection of a particular glycosyltransferase activity. At present a positive reaction is taken to indicate the presence of the appropriate monosaccharide in a terminal or a sub-terminal position in a oligosaccharide side-chain and the identity of the glycosyltransferase is necessarily ambiguous if the identity of the next, proximal monosaccharide is unknown. A negative reaction means that the appropriate monosaccharide is not accessible, but does not mean that it or the appropriate glycosyltransferase are necessarily absent.

The natural functions of lectins are unknown. Most lectins have oligosaccharide side-chains of their own and are classed as glycoproteins (Sharon, N. and Lis., 1972). Thus they are glycoproteins which can combine with other glycoproteins. Mannose, glucosamine, galactose, xylose, and arabinose are the most common monosaccharides of lectins. D-mannose, N-acetyl-D-galactosamine, L-fucose, D-galactose and N-acetyl-D-glucosamine are the monosaccharides which bind with one or more lectins (Sharon, N. and Lis, H., 1972). Aspartic acid serine, and

threonine are particularly abundant in lectins and cysteine and methionine are nearly or completely absent, in most lectins (Sharon, N. and Lis, H., 1972). Lectins range from 26,000 to 400,000 Daltons in molecular weight (Burger, M. M. and Goldberg, A. R., 1967; Burger, M. M., 1969; Gould, N. R. and Scheinberg, S. L., 1970; Marchalonis, I. J. and Edelman, J. M., 1968). Many, if not all, are polyvalent for the monosaccharide they recognize (Sharon, N. and Lis, H., 1972).

I have examined an unusual lectin. The lectin reacts with and agglutinates the erythrocytes of some chickens and some other species, but the reaction with chicken erythrocytes is under genetic and physiological control. The genetic control is simple. Positive animals are either +/+ or +/- and negative animals are -/- (Scheinberg, S. L. and Reckel, R. P., 1962; Durand, L. and Merat, P., 1971). The reaction is restricted to hens, but males show the reaction after treatment with estrogen provided they carry the appropriate allele. The reaction was inhibited by more than one monosaccharide (Reckel, R. P. and Scheinberg, S. L., 1960). I have confirmed the inhibition with one sugar, D-mannose, and have separated the activity into two fractions by isoelectric-focusing and SEPHADEX gel filtration in SEPHADEX. The remarkable features of these molecules are their low apparent molecular weights, about 7×10^3 Daltons, and their thermal stability. These characteristics suggest that these are the simplest of the known lectins, that they should be amenable to complete structural characterization, and that they possess relatively few binding sites.

Materials and Methods

I. Preparation of Saline Extract of Pisum Arvense

Two hundred grams of peas (Pisum Arvense) were soaked in 1000 ml of distilled water overnight. The peas were then homogenized and a clear yellow filtrate was collected via vacuum filtration through no. 1 Whatman filter paper. The pH of the filtrate was adjusted to pH 6.7 with 5M NaOH. The filtrate was acidified after stirring for 1 hour at room temperature by addition of 6M HCl to pH 4.6. The resultant precipitate was filtered out and subsequently discarded. The proteins contained in the clear yellow filtrate were then salted out with solid ammonium sulphate (50% saturation). The precipitate was resuspended in 200 ml of distilled water and dialyzed against distilled water at 4 C until the ammonium sulphate was completely removed. Lastly, the dialyzed extract was lyophilized.

II. Agglutination Assay

In all subsequent experiments, agglutination test were performed as follows. Heparinized blood was obtained from the chickens. The serum was removed by centrifugation at 1500 RPM for 10 minutes in a clinical centrifuge. Following centrifugation, the serum was discarded and the remaining erythrocytes were washed repeatedly in excess volume of saline for at least three times; each wash consisted of suspension of the erythrocytes in excess volume (i.e. 20ml) followed by centrifugation at 1500 RPM for 10 minutes. The triply-washed erythrocytes were then resuspended in a suitable volume of PBS (phosphate-buffered saline) to yield a cell count of 3.00×10^7 cells/ml as determined by a

Fisher autocytometer (cell counter). For test of agglutination, 0.5 ml of cell suspension plus 0.5 ml of pea extract solution (25mg of total lyophilized pea extract/ml of PBS) were placed in 0.5 ml plastic culture tubes. The experimental control consisted of 0.5 ml of cell suspension and 0.5 ml of just PBS. The cell mixtures were then allowed to incubate at 37°C for 1 hour. Following incubation, the cell mixtures were centrifuged at 1600 RPM for 10 minutes. Finally, the cell mixtures were gently resuspended by shaking on a Vortex mixer for 10 seconds. Agglutination was then assessed for each cell mixture both macroscopically (i.e. visual examination) and microscopically. Any clumping of the erythrocytes after gentle resuspension was considered to be indicative of agglutination; arbitrary values (i.e. ++, +++, or ++++) were assigned to each agglutination reading depending on the relative diameter of the cell clump obtained.

III. A Preliminary Survey for Chickens With "Hi" Agglutinogen-bearing Erythrocytes

A preliminary survey for chickens with "Hi" agglutinogen-bearing erythrocytes was undertaken. In this particular experiment, 65 adult white leghorns of known B genotype and 40 adult females of mixed breed were tested for presence or absence of "Hi" agglutinogen-bearing erythrocytes. Five milliliter samples of heparinized blood were obtained from each bird. Preparation of the erythrocytes and agglutination assay were performed as described earlier.

Positive agglutination reactions were observed microscopically using a Leitz Ortholux microscope at 250X magnification. Photomicrographs of typical agglutination reactions were taken with Tri-X Pan

black and white film.

IV. Test of Agglutinability of Leukocytes

Leukocytes were tested for agglutinability by the pea extract. A leukocyte preparation was obtained from bird #1337-38 which was positive for the "Hi" agglutinogen phenotype. Isolation of the leukocytes from 2 ml of heparinized blood was accomplished as follows. Four milliliters of diluted blood (2 ml of whole blood + 2 ml of Alsever's solution) were layered on top of 3.0 ml of Lymphoprep (a commercial preparation of ficoll) in a 10 ml centrifuge tube. The mixture was centrifuged at 1500 RPM for 30 minutes. The resultant white band of leukocytes at the interface between the Lymphoprep and the serum was removed with a Pasteur pipette. The leukocytes were then suspended in 10 volumes of PBS (phosphate-buffer saline, pH 7.0) and centrifuged again at 1500 RPM for 10 minutes. Following centrifugation, the supernatant was removed and saline was added to the cells to resuspend them. Lastly, the cells were washed repeatedly 3 times in saline (each wash consisted of resuspension in 10 ml of saline followed by centrifugation at 1500 RPM). After the final wash, an appropriate volume of saline was added to the leukocyte suspension to yield a cell count of 5.0×10^6 cells/ml as determined by a Fisher autocytometer (Model II). Next, the obtained leukocytes were tested for agglutinability by the pea extract. Half milliliter of pea extract (25 mg/ml in saline) was added to each 0.5 ml suspension of leukocytes. The experimental control consisted of 0.5 ml of saline and 0.5 ml of cell suspension, and the positive control consisted of the same number of "Hi" agglutinogen-bearing erythrocytes from the same donor bird suspended in 0.5 ml of saline + 0.5 ml of the pea extract.

Incubation of the cell mixtures was allowed to take place at 37°C for 1 hour and agglutination was assessed as before.

V. Dilution Study (test of the potency of the pea extract)

To determine the relative strength of the activity of the pea extract, two fold serial dilutions of the extract in 0.5 ml volumes were made in 5.0 ml plastic culture tubes and 0.5 ml (3.0×10^6 cells/ml) samples of "Hi" agglutinogen-bearing erythrocytes from blood donors #26751, #1337, #28229, and #2427 were added to each dilution of the extract. The controls consisted of simple 0.5 ml of saline and 0.5 ml of the same number of erythrocytes in saline. The cell mixtures were incubated at 37°C for 1 hour and agglutination was assessed as described earlier.

VI. Haptenic Inhition Tests

Sugars have been known to inhibit the agglutination of erythrocytes by lectins. In this particular experiment, an attempt was made to find a sugar which would inhibit the agglutination induced by the pea extract. Ten milligram samples of each the following sugars were dissolved in 0.5 ml of pea extract (25mg/ml in saline):

D-melizitose	D-turanose	D-sucrose	
D-fructose	D-trehalose	D-galactose	D-glucose
D-mannose	D-xylose	D-lactose	D-cellobiose
L-rhamnose	D-raffinose	D-ribose	D-lyxose
L-sorbose	D-meliziose	D-arabinose	L-fucose

The resultant sugar-pea extract mixtures were incubated in 0.5 ml plastic culture tubes for 1 hour at 37°C. The experimental controls consisted

of incubating the same sugars (20mg/ml) in 0.5 mls of saline for 1 hour at 37°C. Following completion of the incubation period, 0.5 ml erythrocytes (3.0×10^7 cells/ml in saline) from "Hi" agglutinogen positive bird #1337 was added to each sugar-pea extract mixture and control tubes. Finally, agglutination was assessed as described previously.

VII. Effects of Mannose on the Agglutination Process

Having shown that mannose has an inhibitory effect on the agglutination process induced by pea extract, the effects of different dilutions of mannose on the agglutination were tested. A 0.2M solution of D-mannose was used to make 0.5 ml volumes of two fold serial dilutions ranging from 1 to 1/2048 (dilutions were made with saline). Dilutions of a 0.2M solution of D-fructose, shown to be non-inhibitory to the agglutination process, were also made up. The sugar dilutions were each incubated with 0.5 ml of pea extract (25mg/ml in saline) for 1 hour at 37° C in 5 ml plastic culture tubes. Positive controls consisted of 0.5 ml of saline + 0.5 ml of the pea extract which were also incubated at 37° C for 1 hour. Following incubation, 0.5 ml of "Hi" agglutinogen positive erythrocytes from donor bird #1337 (3.0×10^7 cells/ml in saline) was added to sugar dilution and controls. Lastly, agglutination was assessed as described earlier.

VIII. Effects of Trypsin Treatment on the Agglutination Process

In this experiment, an attempt was made to see whether or not trypsin is capable of exposing or removing the "Hi" agglutinogen receptor sites on "Hi" agglutinogen positive erythrocytes. Trypsin treatment was effected as follows. Ten milliliters of triply-washed erythrocytes from

donor bird #28279 were added to 10 ml of PBS (phosphate-buffered saline, pH 7.0) containing 2.5 mg of trypsin (Difco, 1:250). The "Hi" agglutinogen positive cells were incubated at 37 C for 1 hour. As controls, 10 ml of the same cells were incubated in 10 ml of PBS only for 1 hour at 37 C. After the trypsin treatment, the cell suspensions were washed three times in saline and appropriate volumes of fresh saline were added to the cell suspensions to yield a cell count of 3.0×10^7 cells/ml. Next, .5 ml of pea extract (25mg/ml in saline) was added to each of 5 samples of .5 ml volumes of the trypsin-treated cells and controls. Agglutination was assayed as outlined earlier.

IX. Effects of Temperature on the Agglutination Activity of the Pea Extract

The thermal stability of the pea extract was tested in this experiment. Five .5 ml volumes of the crude pea extract (25mg/ml in saline) were incubated at 37 C, 50 C, 56 C, and 90 C for a duration of 1 hour. Following the incubation period, .5 ml (3.0×10^7 cells/ml) of "Hi" agglutinogen positive erythrocytes from bird donor #14546 was added to each of the heat-treated pea extract. The controls consisted of .5 ml of the same cell suspension plus .5 ml of untreated pea extract. Finally, the cell mixtures were tested for agglutination as described earlier.

X. Effects of Neuraminidase on the Agglutination Process

In this particular experiment, the possible role of sialic acid residues in the agglutination induced by the pea extract was examined using neuraminidase. Two fold serial dilutions of neuraminidase (from Cl.perfringens, Type VI obtained from Sigma Chemical Co.) ranging in

activity from 1/2 Unit/ml to 1/2048 Unit/ml were made up with PBS (phosphate-buffered saline, pH 5.7) in 0.5 ml volumes. Next each enzyme dilution was added to 0.5 ml of "Hi" agglutinogen positive erythrocytes (3.3×10^7 cells/ml in PBS, pH 5.7) obtained from bird donor #14546. As experimental controls, 0.5 ml of PBS, pH 7.0 was added to 0.5 ml samples of the same cell suspension. Two other controls were also used: negative control no. 1 consisted of 0.5 ml of erythrocytes plus 0.5 ml of neuraminidase (1/2 Unit/ml) and control no. 2 consisted of 0.5 ml of PBS (pH 5.7) plus 0.5 ml of the same cell suspension. All the cell suspensions were incubated for 1 hour at 37°C. Next, the cell suspensions were washed three times in saline to remove the released sialic acid residues and neuraminidase. Following the saline washes, the cell suspensions were made up to 0.5 ml again with fresh saline. Lastly, 0.5 ml of pea extract (25mg/ml in saline) was added to all cell suspensions except those of control no. 1 and control no. 2. Agglutination was assayed as described earlier.

XI. Effects of pH on the Agglutination Process

The effects of pH on the agglutination process were investigated in this experiment. Phosphate buffers (.2M) of the following pH's were prepared: pH 5.7, pH 6.0, pH 6.5, pH 7.0, and pH 8.0. Samples of 0.5 ml volumes of pea extract (25mg/ml in buffered PBS) were added to 0.5 ml volumes of each of the different phosphate buffers to 0.5 ml plastic culture tubes. Next, 0.5 ml volumes of "Hi" agglutinogen positive cells from donor #14546 were added to each of the phosphate buffer-pea extract mixtures. The experimental controls used in this study consisted of two controls: positive control no. 1, consisting of 0.5 ml

of PBS (pH 7.0) + 0.5 ml of pea extract + 0.5 ml of erythrocytes and negative control no. 2, consisting of 0.5 ml of cells + 1.0 ml of the buffer of the pH being tested. Incubation of the cell mixtures was allowed to continue for 1 hour at 37°C. Following incubation, agglutination was assessed as described earlier.

XII. Test for Species Specificity

The specificity of the agglutinating activity of the pea extract was tested in this experiment. Heparinized blood samples were obtained from the following animals: trout, human, coyote, ground squirrel, rabbit, and newt. The blood samples were prepared and washed as described in the agglutination assay. Appropriate volumes of saline were added to each triply-washed suspension of erythrocytes to yield a cell count of 3.4×10^7 cells/ml. Pea extract in 0.5 ml volumes (25 mg/ml in saline) was added to 0.5 ml volumes of each cell suspension in plastic culture tubes and agglutination was tested as described before. As controls, "Hi" agglutinogen positive cells from donor bird #14546 were also reacted in 0.5 ml volumes with 0.5 ml volumes of the pea extract (25mg/ml in saline) and the negative controls consisted of 0.5 ml of saline + 0.5 ml of the "Hi" agglutinogen positive cells (3.4×10^7 cells/ml).

XIII. Test for Cell Electrophoretic Mobility Differences

The question as to whether or not the presence of the "Hi" agglutinogen receptors on the cell surface endows the erythrocytes with a different electrophoretic mobility or cell surface charge was posed in this experiment. Blood samples (heparinzed) were obtained from "Hi" agglutinogen positive donors #14546 and #14428 and two "Hi" agglutinogen

negative bird donors #1333 and #13277. The blood samples were centrifuged at 1500 RPM to remove the serum and the erythrocytes were washed three times in saline as described previously. Electrophoretic mobility measurements were made for each of the erythrocyte samples using a microelectrophoresis apparatus described by Bangham, A. D. et al, 1958. The electrophoretic measurements consisted of measuring the time in seconds required by a single cell to traverse a 45 micron path in the electrophoretic field. Twenty-three such measurements were made for each blood sample. The electrophoretic conditions used were as follows:

voltage	=	40 volts D. C.
temperature	=	25 C
migration path	=	45 microns
cell suspension medium	=	.145 M NaCl

XIV. Fluorescence Microscopy of "Hi" Agglutinogen-bearing Cells

The purpose of this experiment was to demonstrate that the "Hi" agglutinogen is located on the cell surface of the reactive erythrocytes. This experiment was carried as follows. First of all, a fluorescein conjugate of the pea extract was prepared as follows. Five milliliters of pea extract (20mg/ml in distilled water) were reacted with 5 mg of fluorescein isothiocyanate in a carbonate-bicarbonate buffer, pH 9.0 (.5M) and 4 ml of .15M NaCl solution at 4 C. The mixture was left stirring at 4 C for 18 hours. Next, the mixture was dialyzed against several changes of phosphate-buffered saline (pH 7.0) until the dialyzing fluid no longer showed fluorescence. The conjugate was lyophilized and stored at -20 C in a small vial.

Once the fluorescein conjugate of the pea extract was obtained,

the "Hi" agglutinogen positive erythrocytes of bird #14546 and #14428 were subjected to fluorescence microscopy as follows. Five milliliters of blood was obtained from each "Hi" agglutinogen positive bird and as controls, 5 ml of blood was collected from each of the two "Hi" agglutinogen negative birds (#1333 and #13277). The blood samples were washed three times in saline. Next, blood smears of each blood sample were made by spreading very rapidly, a drop of cells across a microscope slide with the aid of another slide. After drying the smears, the slides were fixed in 95% ethanol for 1 minute, drained, dipped in phosphate-buffered saline, and air dried. After the smears were thoroughly dried, they were then covered completely with the fluorescein-pea extract conjugate (100mg/ml in PBS, pH 7.0). As controls for autofluorescence, some smears were covered with pea extract only in the same concentration. All slides were next incubated for 1 hour at 37 C. Following the incubation period, the slides were washed in three changes of phosphate-buffered saline and one final change of distilled water for 5 minutes each. Next, the slides were mounted with buffered glycerol (9 parts glycerin: 1 part phosphate buffered saline) and subjected to fluorescence microscopy.

The prepared slides were examined under a Leitz Ortholux microscope equipped with optics for ultra-violet, phase, and transmitted light microscopy. Ultra-violet illumination was supplied by two HBO 200 lamps in separate housings to provide both transmitted and incident excitation of fluorescent specimens. It was found that incident excitation gave the best fluorescence intensity, and hence most of the prepared slides were examined under incident excitation. Photomicrographs were taken of fluorescent cells using Kodak High Speed Ektachrome colour slide film (ASA 160). The exposure times required ranged from 10 seconds to 120

seconds depending on the intensity of the observed fluorescence.

XV. Determination of the Total Protein Content of the Pea Extract

As an initial attempt at characterizing the pea extract preparation, the total protein content was determined using the biuret method. Using this method, protein measurements were made as follows. Samples of the pea extract in 1 milligram quantities were dissolved in 1.0 ml of distilled water and incubated with 8 ml of biuret reagent (obtained from Fisher Scientific Company) at room temperature for 30 minutes. At the end of the incubation period, the optical densities of the resultant colours were determined on a Spectronic 20 spectrophotometer at 550 nm. In order to obtain a standard calibration curve, bovine serum albumin (Crystalline, from Nutritional Biochemicals Corporation) was made up in the following dilutions:

0 mg/ml in distilled water, 5 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml and 25 mg/ml.

One milliliter samples of each albumin concentration were reacted with 8 ml of biuret reagent and the optical densities were measured. A graph of optical density as a function of protein concentration was compiled to permit graphical estimates of the total protein contents of the pea extract samples.

XVI. Determination of the Total Carbohydrate Content of the Pea Extract

The total carbohydrate content of the pea extract was estimated in this experiment using a ferricyanide method (Keleti and Lederer, 1974). Samples of pea extract (lyophilized) in .5 mg to 1.0 mg quantities were added to 1.0 ml of carbonate-cyanide reagent (1.33 g Na_2CO_3 + 162.5 mg

KCN + distilled water to yield a final volume of 250 ml) and 1.0 ml of ferricyanide reagent (125 mg $K_3Fe(CN)_6$ + distilled water to yield a final volume of 250 ml). The mixtures were thoroughly shaken and subsequently placed in a boiling water bath for 15 minutes. After cooling to room temperature, 5.0 ml of ferric-iron reagent (375 mg $FeNH_4(SO_4)_2$ + 250 mg sodium lauryl sulphate + 0.05N H_2SO_4 to a final volume of 250 ml) were added to each sample. The samples were allowed to stand at room temperature for 15 minutes. Lastly, the optical densities of the samples were read at 690 nm on a Spectronic 20 spectrophotometer. In order to obtain a standard calibration curve, dilutions of glucose ranging in concentration from 0 microgram/ml to 2.0 microgram/ml were prepared and treated as described for the pea extract samples. A graph of optical density as a function of carbohydrate (glucose) concentration was compiled to allow graphical estimates of the total carbohydrate content of the pea extract samples.

XVII. Qualitative Disc Gel Acrylamide Electrophoresis of the Pea Extract

The crude pea extract was subjected to disc gel acrylamide electrophoresis to obtain a general picture of the minimum number of protein components that is present in the extract. Samples of 20 microliter volumes of the pea extract (10mg/ml in distilled water) were mixed with equal volumes of 40% sucrose solution and layered on top of acrylamide gel columns which had been prepared according to the method of Davis (1964). Electrophoresis was performed under the following conditions:

1. electrophoresis buffer - Tris-glycine, pH 8.3
   (consisting of 1.2 grams of tris (hydroxymethyl) aminomethane + 5.76 grams of glycine + distilled water to yield a final volume of 2.0 litres).
2. voltage - 300 volts pulsed D. C., 300 pulses/sec., 1.0 mFd., and 30 milliamperes provided by a 4100 Pulsed Constant Power Supply (Ortec Corporation).
3. duration - 1 hour at room temperature.

After the electrophoretic run, the gel columns were removed from the glass tubes and stained in 7% acetic acid containing .2% Buffalo Black (Amido Schwartz). Following the staining procedure, the gel columns were destained for several days in 7% acetic acid in order to remove the excess unbound dye. Lastly, the destained acrylamide gel columns were scanned densitometrically by a Photovolt densitometer (Photovolt Corporation).

XVIII. Isoelectricfocusing of the Crude Pea Extract

An attempt was made to isolate the active agglutinins from the crude pea extract on a preparative scale using an isoelectrofocusing technique. A 10 ml sample of pea extract (10mg/ml in distilled water) was electrofocused as follows. A linear sucrose density gradient (total volume 440ml) was first prepared with the aid of a LKB Ampholine Gradient Mixer (LKB-Produkter, Sweden) using the following gradient solutions:

1. dense gradient solution -

   sucrose - 107.5 g

   volume of

   H_2O + 7.0 ml of Ampholine - 150 ml

   total volume - 215 ml

   concentration of sucrose - 50%(W/V)

2. light gradient solution -

   sucrose - 10.75 g

   volume of H_2O + 3.75 ml

   of Ampholine - 207 ml

   total volume - 215 ml

   concentration of sucrose - 5% (W/V).

The Ampholine used to establish a pH range of 3.5 - 10.0 was a sterile 40% solution of carrier ampholytes (from LKB, Sweden) with a pH-range of 3.5-10.0; the final concentration of ampholytes in the sucrose density gradient was 1%. The sucrose density gradient was set up in a LKB 8100 440ml isoelectrofocusing column (LKB, Sweden). The pea extract sample (100mg total) was added to the dense gradient solution during preparation of the sucrose density gradient. The electrode solutions employed for the electrophoretic run were as follows:

1. cathode electrode solution - (pH 11.7)

   48.0 g of sucrose + 30 ml of H_2O + 20 ml of 1M NaOH

2. anode electrode solution - (pH 2.0)

   6 ml of 1M H_3PO_4 + 34 ml of H_2O

The isoelectrofocusing column was set up in a refrigerated walk-in cold room and electrophoresis was performed under the following experimental conditions:

1. current - initial = 30 milliamperes; final = 0.5 milliamperes
2. voltage - initial = 300 volts; final = 1200 volts
3. power - initial = 9.0 Watts; final = 6.0 Watts
4. temperature of run = 4 C
5. total duration of run - 24 hours

The electric current was supplied by a model 3371E D.C. Power Supply (LKB, Sweden). Following completion of the electrophoretic run, a total of 70 fractions of 40 drops each (approximately 5.0 ml) was collected from the bottom of the column via a draining outlet, using a LKB fraction collector. The absorbance of each fraction was measured at 280 nm in order to obtain a protein elution profile. The pH of each of the 70 fractions was also measured to yield a pH profile of the separated fractions.

The last step of this iselectrofocusing experiment consisted of testing the resultant six major protein fractions for agglutinating activity. Half milliliter samples of each of the peak fractions were added to .5ml of "Hi" agglutinogen positive cells obtained from bird donor #14546 in 5 ml plastic culture tubes. As controls, .5 ml of saline was added to .5 ml of the same cell suspension (3.2×10^7 cells/ml in saline). The mixtures were incubated at 37 C for 1 hour and agglutination was assessed as described previously.

XIX. Sucrose Density Gradient Centrifugation Fractionation of the Pea Extract

In this experiment, the crude pea extract was fractionated by sucrose density gradient centrifugation. Samples of 5 mg of pea extract (dissolved in .5ml of distilled water) were layered on top of four 15-35% continuous sucrose density gradients (35ml volume, total)

which were prepared beforehand in cellulose nitrate tubes (1" dia. X 3 1/2", Beckman Instruments Inc.), using a Beckman density gradient former (Beckman Instruments Inc.). High speed centrifugation was performed in a Beckman L2-65B Ultracentrifuge using a swinging bucket rotor (SW-27, Beckman Instruments Inc.). A speed of 25,000 RPM was maintained for 20 hours at a running temperature of 4 C. Following centrifugation, 20 drop fractions (approximately 2 ml) were collected from the bottom of each gradient. The obtained fractions were pooled and the absorbance of the pooled fractions was measured at 280 nm on a Beckman Du-2 spectrophotometer in order to obtain an elution profile.

Lastly, the major peak protein fractions were tested for agglutination activity. Half milliliter samples of each peak fraction were added to .5 ml of "Hi" agglutinogen positive erythrocytes donated by bird #14546 (3.2×10^7 cells/ml in saline) in 5 ml plastic culture tubes. The controls consisted of .5 ml of saline plus .5 ml of cells. The cell-protein fraction mixtures were incubated at 37 C for 1 hour and agglutination was assessed as described earlier.

XX. Fractionation of the Pea Extract Via Sephadex Gel Column Chromatography

Sephadex gel column chromatography was resorted to in this experiment. A Sephadex gel column (3 cm internal dia. X 45 cm) was set up using Sephadex G-25 (Pharmacia Co.). The Sephadex gel was soaked for three days in .85% NaCl containing 0.001% sodium azide and packed into the column at 4 C. Prior to use, the packed column was eluted with 2 liters of .85% NaCl in order to allow the gel to equilibriate and eliminate contaminants from the column. A 10 mg sample of crude pea extract (dissolved in 2.0 ml of .85% NaCl) was applied onto the column with a

syringe and elution was effected at 4 C with .85% NaCl buffer. Five milliliter fractions were collected (approximately 50 drop fractions) with the aid of a LKB fraction collector (LKB, Sweden). The absorbance of each fraction was measured at 280 nm in order to obtain an elution profile .

Lastly, the major protein peak fractions were tested for agglutinating activity. Half milliliter samples of each peak fraction were added to .5 ml of "Hi" agglutinogen positive cells (donor #14546) in 5ml plastic culture tubes. As controls, .5 ml of saline was added to .5 ml of the same cell suspension (3.3×10^7 cells/ml in saline). The cell mixtures were incubated at 37 C for 1 hour and agglutination was assessed as described previously.

Ten microliter samples of the major protein peak fractions and crude pea extract (10 mg/ml in distilled H_2O) were also subjected to disc gel acrylamide electrophoresis according to the technique described previously under the following electrophoretic conditions:

1. electrophoresis buffer - Tris-glycine, pH 8.3
2. current - 300 volts pulsed D.C., 300 pulses/sec., 1.0 mfd., and 30 milliamperes (supplied by an Ortec Pulsed Constant Power Supply)
3. running temperature - room temperature (23 C)
4. duration of run - 1 hour

At the completion of the electrophoretic run, the gels were removed from the glass tubes and stained in 7% acetic acid containing .2% Buffalo Black and destained for several days in 7% acetic acid with frequent changes of the destaining solution. Finally, a photograph of the destained gels was taken to record the protein bands observed.

XXI. Estimation of the Molecular Weights of the Active Components

The agglutination active Peaks IV and VI from the isoelectric-focusing experiment were subjected to thin-layer gel filtration in order to obtain estimates of their molecular weights. Using procedures similar to those of Radola (1968), estimations of the molecular weights of Peaks IV and VI were carried out as follows. A thin layer of Sephadex gel (0.8 mm thick) was prepared on a 20 cm X 40 cm glass plate by spreading a slurry consisting of 9.0 grams of Sephadex gel G-200 (Pharmacia Co.) in 200 ml of phosphate-buffered saline. The resultant gel layer was placed in a thin-layer gel chromatographic chamber (Pharmacia Co.) and allowed to equilibrate overnight with the eluting buffer (PBS). Once equilibrated, 20 microliter samples of Peaks IV and VI and other protein standards with known molecular weights were applied 1.5 cm from the upper end of the gel layer. The protein standards used were aldolase, chymotrypsinogen A, ferritin (from horse spleen), and ribonuclease A. Next, the two ends of the gel layer were connected to the eluting phosphate-buffered saline reservoirs via strips of Whatman #1 filter paper measuring 20 cm X 5 cm. The gel layer was tilted at an angle of 30° from the horizontal and descending gel filtration was allowed to proceed at room temperature for 9 hours. At the end of the run, a duplicate of the gel layer was obtained by layering a sheet of Whatman #1 chromatography paper (20 cm X 40 cm) gently on top of the gel layer. Using this technique, all the resolved protein samples were completely transferred from the gel onto the paper without any changes in their migration positions. The paper duplicate was next stained for 15 minutes in Ponceau S and destained in 7% acetic acid. Following de-staining, the migration distance (from the origin)

was measured for each sample and the relative mobility (i.e. relative to ferritin) of each sample was calculated as follows:

$$\text{Relative Mobility} = \frac{\text{migration distance of sample}}{\text{migration distance of ferritin}}$$

Lastly, a graph of relative mobilities versus log of molecular weights was plotted to allow interpolation of the molecular weights of Peaks IV and VI. In order to obtain average molecular weight estimates, two such gel filtration trials were performed.

Results

The agglutinating activity of extracts of Pisum arvense was tested with erythrocytes from two kinds of chickens. The first was drawn from the mixed breed for which Durand and Merat (1971) described the agglutination of erythrocytes by extracts of Lens culinaris. Two of 38 adult females were positive. The second population was drawn from White Leghorns whose B Blood type was known. The adult males were negative. Six of the 42 adult females were positive (Table 1). There was no indication of an association of the positive reaction with any of the seven B alleles represented in this population (Table 1a). On the basis of earlier information that the Hi agglutinogen is widely distributed among all breeds and can be detected with extracts of either Pisum arvense or Len culinaris (Scheinberg, S. L. and Reckel, R. P., 1961b) it was assumed that the "Hi" agglutinogen of the White Leghorns is identical with that which Durand and Merat (1971) had described and for which they had confirmed the mode of inheritance as described by Scheinberg and Reckel (1962).

The classification of chickens as Hi positive or negative was done by incubating 1.5×10^7 erythrocytes with 12.5 mg protein of extract in a final volume of 1.0 ml of saline at 37 C for 1 hour. Under these conditions the reactions are unambiguous (Figure 1). The effect of diluting the extract was tested with erythrocytes from four Hi positive chickens (Figure 2). Arbitrary classification of the reactions according to the size of the agglutinates demonstrates a reduction in the reaction when the extract is diluted to 1.56 mg protein per ml and a disappearance of the reaction when the extract is diluted to 0.10 mg protein per ml.

Leukocytes from the blood of one Hi positive chicken were mixed with extract under conditions comparable to those used to demonstrate the presence of the Hi agglutinogen on the erythrocytes. These tests did not differ from those in which saline was used in place of extract and are regarded as negative.

The reaction with erythrocytes was used to test the inhibition of lectin-binding by free monosaccharides and oligosaccharides. Eleven monosaccharides, six disaccharides, and two trisaccharides were tested. The monosaccharides were D-arabionse, D-fructose, L-fucose, D-galactose, D-glucose, D-lyxose, D-mannose, D-ribose, L-rhamnose, L-sorbose, and D-xylose. The disaccharides were D-cellobiose, D-lactose, D-melibiose, D-trehalose, D-sucrose, and D-turanose. The trisaccharides were D-melizitose, and D-raffinose. A mixture of sugars was also tested. Only the mixture and D-mannose inhibited the agglutination. This confirms an earlier report that D-mannose inhibits the agglutination but fails to confirm the report that the monosaccharide, D-fructose, and the disaccharides, D-sucrose, D-trehalose, and D-turanose and the trisaccharide, D-melizitose are inhibitory (Reckel, R. P. and Scheinberg, S. L., 1960). The two monosaccharides were compared (Figure 3). D-fructose was non-inhibitory at all concentrations tested. D-mannose was inhibitory at a final concentration of 6.25mM. Inhibition was greater at higher concentrations, but complete inhibition was not seen at 200 mM, the highest concentration tested. Mannose is not a constituent of any of the disaccharides or trisaccharides tested.

Trypsin is known to degrade cell surfaces (Kornfeld, S. and Kornfeld, R., 1969; Winzler et al., 1967) and is a conventional means of enhancing the agglutination of cells treated with antibodies. Hi

positive erythrocytes from one chicken were treated with trypsin as a test of the susceptibility of Hi agglutinogen to alteration by proteolytic enzymes. The agglutination was neither enhanced nor reduced. If the Hi agglutinogen was partly removed by trypsin, the loss must have been compensated by increased susceptibility to agglutination.

Some lectins are sensitive to temperature. An extract of Pisum arvense was incubated at 37, 50, 56, or 90 C for one hour prior to testing its reaction with Hi positive erythrocytes at room temperature (Figure 4). The agglutination decreased as the temperature of preincubation increased, but was not abolished. Compared to other lectins the lectin activity of Pisum arvense is relatively stable.

Neuraminidase is used to enhance or depress some cell surface reactions (Burger, M. M. and Goldberg, A. R., 1967; Uhlenbruck, G. and Winzler, G., 1970). Enhancement is usually interpreted as evidence that sialic acid residues shield the reactive site of the receptor. Depression may be interpreted as evidence that sialic acid is part of the reactive site of the receptor or it may mean that sialic acid residues act in a secondary manner to favour agglutination initiated at a reactive site which does not include sialic acid. Treatment of Hi positive erythrocytes from one chicken depressed the agglutination. The depression was a function of the concentration of neuraminidase (Figure 5).

The sensitivity of Hi agglutinogen to modest variation of pH was tested (Figure 6). The erythrocytes of one chicken were mixed with a concentration of extract which causes strong agglutination. No effect was seen. The possible effect on weak agglutination was not

tested.

The biological selectivity of Hi agglutinogen was tested with erythrocytes from representatives of several species. Human erythrocytes of type O and rabbit erythrocytes agglutinated as strongly as Hi positive chicken erythrocytes (an agglutination score of 4 was obtained for all three cases). Coyote (Canis tatrans) erythrocytes showed intermediate agglutination (an agglutination score of 3) and ground squirrel (citettus richardsoni) erythrocytes showed weak agglutination (an agglutination score of 1). Neither newt (Triturus Triturus) nor trout (Salmo ctavki) erythrocytes showed any agglutination. The susceptibility of these agglutinations to the presence of D-mannose was not tested.

The erythrocytes of two Hi positive females and two Hi negative males were compared electrophoretically as a test of the possibility that Hi agglutinogen is associated with a gross difference in cell surface charge, i.e. one which greatly exceeds possible other causes. Each sample of erythrocytes was tested 23 times and the variation in these tests was analyzed for differences between repetitions and for differences between individuals (Table 2). The differences between repetitions were significant ($p < 0.025$) and the differences between individuals were non-significant ($0.100 > p > 0.050$). The first tests varied more than the others and were removed from the analysis. This reduced the differences among tests to non-significance. The mean differences among individuals remained non-significant ($0.100 > p > 0.050$). The greatest source of variation in the remaining data were the repetitions for one Hi positive female; the other Hi positive female gave the least variation. The analysis does not demonstrate a difference between Hi positive females and Hi negative males, but it

does suggest that a difference might be found if the test was repeated on a larger scale. It is not possible to infer from these data whether such a difference would appear as due to sex, Hi, or unknown individual factors.

An extract was treated with fluorescein isothiocyanate, the free fluorescein was removed by dialysis, and the labelled extract was incubated with Hi positive erythrocytes (Figure 7). The Hi positive erythrocytes fluoresced brightly. The fluorescence was uniformly distributed over the cell surface of the Hi positive erythrocytes. No such fluorescence pattern was observed in the Hi negative erythrocytes.

The protein content of one extract was estimated as 82 percent of the dry weight. Presumably the amount of this protein which has lectin activity is relatively small, but the amount of total proein corresponds to that of other active extracts (Toms, G. C. and Western, A., 1971), that for Glycine max (88 percent) and Phaseolus vulgaris (81 percent).

Lectins are presumed to be glycoproteins largely because of the protein-bound carbohydrate found in active extracts. The extract from Pisum arvense contains 4 percent carbohydrate which is the percent of carbohydrate in extracts of Phaseolus lunatus (lima bean), Phaseolus vulgaris (black kidney bean), Triticum vulgaris (wheat), and Agaricus campestris (meadow mushroom), but higher than that for Pisum sativum (Sharon, N. and Lis, H., 1972), 0.3 percent. The Hi agglutinin is likely to be a glycoprotein since this term is applied to proteins which are 1 to 80 percent carbohydrate (Spiro, R. G., 1973), but no direct proof that the carbohydrate is bound to or complexed with the protein was attempted.

The molecular homogeneity of the agglutinating activity was

examined by sucrose density gradient centrifugation (Figure 8). The first five of the seven peaks identified by absorbance at 280 nm contained activity. The location of activity in the first fractions implies that the activity is found in the larger proteins. The number of active peaks could be an artifact of diffusion which is difficult to control. Homogeneity was further examined by passing crude extract through SEPHADEX G-25 selected because the other grades of SEPHADEX absorb plant lectins very strongly (Entlicher et al., 1969). One distinct peak containing most of the activity was followed by two smaller, possibly artifactual peaks (Figure 9). This suggested that the activity is associated with proteins of modest size, in contrast to the impression obtained from sucrose density gradients. The three peaks were examined separately by acrylamide electrophoresis (Figure 13). The second and third peaks did not show bands and are presumed to have been too dilute. The first peak gave a banding pattern which seemed to correspond to a dilution of crude extract. This implies that the extract is electrophoretically heterogeneous, having 10 or 11 distinct mobility classes (Figure 15), but is relatively homogeneous with respect to molecular size.

The electrophoretic heterogeneity was examined more critically by isoelectricfocusing. This resolved six peaks (Figure 14). Activity was found in Peaks IV and VI. The isoelectric points are pH 8.2 and 7.4. These two peaks were subjected to SEPHADEX gel filtration in a thin layer. Both peaks have molecular weights near 7 X 10^3 Daltons (Figures 10, 11, and 12). The estimates for peak IV are 6910 and 7080 and the estimates for peak VI are 7080 and 7944. The second estimates are 11 percent greater than the first estimates.

DISCUSSION

The discovery toward the end of the last century that animals produce substances which agglutinate the erythrocytes of other animals and the realization that these agglutinating substances were similar to agglutinins produced by immunization led to a search for protective substances in plants. Extracts of some seeds, particularly the seeds of legumes, were found to have agglutinins for human and other erythrocytes. There is a selectivity associated with the source of the agglutinin and the source and type of the erythrocyte. The agglutinins clearly differ from these induced by immunization of higher vertebrates. They are natural and invariant products whose reactions are remarkably consistent. This is in contrast to induced antibodies which vary from one antiserum to another and which we now know to be mixtures of proteins of differing specificity and structure. Although antibodies and plant extracts may share the ability to discriminate between very similar, but different, structures by binding more strongly to molecules of one configuration, the binding sites of plant extracts appear to be smaller and to be delineated more exactly. All plant agglutinins appear to recognize single monosaccharide molecules, or single monosaccharide constituents of larger molecules, independent of the presence of neighbouring molecules, or constituents, except as these may hinder access to the target monosaccharide. Such steric hindrance seems common because all cell surfaces are presumed to have some of the target monosaccharides as constituents of larger molecules, yet each plant extract fails to react with many kinds of erythrocytes and other cells. Sequential degradations and symtheses of human blood group substances indicate that the plant extracts react with

terminal monosaccharide constituents of oligosaccharide chains whether the monosaccharide terminates an axial chain or side chain of a branched oligosaccharide. The agglutination of erythrocytes appears to be due to unexplained alterations of the cell surface secondary to this binding to terminal constituents of the carbohydrate side-chains of glycoproteins. The identity of the terminal group is usually inferred from the identity of the free monosaccharide which is most effective as an inhibitor of the agglutination. This general interpretation also applies to extracts of lower animals which have agglutinating properties comparable to those of extracts of plants.

The term "lectins" encompasses all natural proteins which selectively combine with monosaccharide, either free or as a constituent, without evidence of enzymatic action comparable to that which accompanies such naturally occurring reactions as the combination of lysozymes with the bacterial cell wall. The secondary effects of the combination of lectins with cell surfaces may be equally profound, but they have interpreted as representing an enzymatic activity of the lectin. Lectins are not known to play a protective role in either plants or lower animals and their natural role is as great a mystery as ever.

Lectins have technical value because of their selectivity and consistency. They can be used to detect the presence of an accessible, presumably terminal, monosaccharide constituent of a glycoprotein component of the cell surface. The reaction leads to the inference that the cell possesses a glycosyltransferase which attaches the target monosaccharide to the carbohydrate side-chain of the glycoprotein. Since the identity of the monosaccharide immediately proximal to the

target is usually unknown, the identity of the glycosyltransferase is usually unknown. A non-reaction leads to the inference that this glycosyltransferase is absent or cannot act because another glycosyltransferase which acts earlier in the construction of the side-chain is absent. Another, as yet untested possibility is that a glycosyltransferase on another cell normally adds the target monosaccharide to the test cell, and hence, a nonreaction represents a defect in this unknown cell, which might be a fixed constituent of the tissues in which the erythrocyte matures. These biochemical uncertainties do not, however, impair the use of lectins as genetic tools when the reaction with lectin follows simple genetic rules. Such is the basis for the reaction of lectins from Pisum arvense and Lens culinaris with the erythrocytes of some mature female chickens.

I have examined the reaction with chicken cells in order to determine its specificity which has not been examined since the original report. I did not re-examine the inheritance of the reaction since this has been confirmed (Durand, L. and Merat, P., 1971). I obtained stock used in the genetic confirmation by Durand and Merat (1971) and demonstratedthe reaction of the erythrocytes of two individuals with an extract of Pisum arvense. There is, therefore little doubt that the reaction with erythrocytes of local B-genotyped chickens is the same reaction as that originally described as "Hi" by Scheinberg and Reckel (1961 b).

The Hi agglutinin of Pisum arvense agglutinates the erythrocytes of some mature female chickens. The genetically controlled variation of this reaction should permit the reaction to be used as a marker for detection of co-inheritance with other characteristics. Before it can be used this way the basic features of the reaction should be better

known. The reaction is inhibited by D-mannose, but not by other carbohydrates which Reckel and Scheinberg (1960) described as inhibitory. In fact, the variety of inhibitors they described was remarkable. Lectins are relatively specific and their description was not in keeping with expectation. The fact that only D-mannose proved inhibitory makes the Hi system much more acceptable. The decrease in agglutination with dilution of the Hi agglutinin and the decrease in inhibition with dilution of D-mannose are linear which is what we would expect for a reaction initiated by combination of a lectin with D-mannose.

Isoelectricfocusing shows that the Hi agglutinating activity occurs in two differently charged molecules. The migration of these in thin layer SEPHADEX suggests that both are large polypeptides rather than proteins. If the estimates of molecular weights 7×10^3 Daltons are reasonably accurate, the Hi lectins are about one half as large as the variable region of a polypeptide chain of an immunoglobulin. It is possible that the migration in SEPHADEX is misleading because some grades of SEPHADEX bind lectins quite strongly (Entlicher et al., 1969). There are two observations which tend to support the SEPHADEX estimates. The Hi agglutinating activity is relatively stable at temperatures above $37^{\circ}C$, which implies a high degree of internal cross-linking, a high net positive charge, or small size. The degree of cross-linking cannot be estimated, but the isoelectric points rule out the second alternative. The second observation consistent with small size is the fact that the lectins isolated by isoelectricfocusing were submitted to hollow fibre dialysis with fibres whose cut off points are equivalent to 5×10^3 Daltons. Thus the estimated molecular weights, for both lectins, of 7×10^3, are not inconsistent with the steps taken.

The dialysis would, however, have retained larger molecules better than small ones and cannot be interpreted as proving that the molecular weight is as low as 7×10^3 Daltons. I conclude that the slow migration in SEPHADEX should be re-examined to learn if it represents binding to SEPHADEX or low molecular weight. If the latter alternative proves correct it means that the lectins of Pisum arvense are the smallest lectins yet described and raises a serious question about our present interpretation of protein-protein aggregation in the cell surface. It should be noted that the attempts to separate Hi agglutinating activity by centrifugation in sucrose density gradients did not indicate that the activity was restricted to proteins of small molecular weight.

Chapter 2

Alkaline Phosphatase

Introduction

Law and Munro (1965) demonstrated the presence of two mutually exclusive forms of alkaline phosphatase in the plasma of chickens. Using starch gel electrophoresis, the two phenotypes observed were variant F, a rapidly migrating band between the albumin and transferrin regions and variant S, a slower migrating band which appeared in the transferrin region. The alkaline phosphatase phenotypes may be represented as follows:

Analyses of pedigreed families revealed that the fast phenotype is determined by a single autosomal dominant gene which is allelic to a recessive gene responsible for the slow migrating phenotype S. The authors assigned the symbols $\underline{Ap}^{2}$ and $\underline{Ap}^{4}$ to the genes for fast and slow forms, respectively. In a heterozygous individual, $\underline{Ap}^{2}$ excludes the appearance of the product of the $\underline{Ap}^{4}$ allele. Consequently, chickens having the F phenotype are either homozygotes ($\underline{Ap}^{2}/\underline{Ap}^{2}$) or heterzygotes ($\underline{Ap}^{2}/\underline{Ap}^{4}$).

The work of Law and Munro was later confirmed by Wilcox (1966) who claimed that the mode of inheritance of the alkaline phosphatase isozymes observed in his investigation was in agreement with that proposed earlier by Law and Munro. Further confirmation was provided by Csuka and Petrovsky (1972).

In 1970, Tamaki and Tanabe observed a new alkaline phosphatase phenotype consisting of a fast migrating band and a slow migrating band in addition to the previously mentioned S and F phenotypes. Strangely enough, this phenotype had never been reported by Law and Munro, nor by other workers who later confirmed their findings.

The purpose of this study was to resolve whether or not only two phenotypes of alkaline phosphatase exist as claimed by Law and Munro (1965). Resolution of this question was felt to be a necessity before any attempts to confirm the genetics of this marker could validly proceed.

Materials and Methods

Serum samples were collected from 26 adult chickens whose B genotype was known. Ten microliter volumes of each sample were subjected to disc gel acrylamide (5 percent) electrophoresis according to the techniques of Smith et al. (1965). The electrophoretic conditions employed were:

1. Tris-borate buffer, pH 9.5
2. 300 volts pulsed D. C., 300 pulses/sec., 1.0 mfd., and 20 milliamperes.
3. duration of electrophoretic run - 35 minutes.

Following completion of the electrophoretic run, alkaline phosphatase bands were detected by the staining technique of the same authors (Smith, I. et al., 1965). The electrophoretic mobility of each observed alkaline phosphatase isozyme relative to the added bromophenol blue dy marker was determined and expressed as percent mobilities.

Results

The alkaline phosphatase phenotypes observed were contrary to what was reported by previous investigators (Table 3) (Law, G. R., and Munro, S. S., 1965; Wilcox, F. H., 1966; Csuka, J. and Petrovsky, E. 1972). A single slow-migrating band (S) with a mean relative electrophoretic mobility of 50 percent (i.e. relative to the electrophoretic mobility of an added marker dye, bromophenol blue), a fast-migrating band (F) with a mean mobility of 60 percent and a third phenotype consisting of two bands (D) were observed (Figure 16). The distribution of the three phenotypes is presented in Table 3. No associations between the tested B genotypes and alkaline phosphatase phenotypes are apparent.

Discussion

Genetically determined protein variants can differ by carbohydrate components -- neuraminic acid, for example. The alkaline phosphatase isozymes of the chicken seem to provide support for this statement. Law (1967) reported that after neuraminidase treatment, the fast-migrating alkaline phosphatase isozyme was retarded in electrophoretic mobility to a point similar to the slow-migrating isozyme; the electrophoretic mobility of the slow-migrating isozyme was unaltered after neuraminidase treatment. These findings strongly suggest that the alkaline phosphatase isozymes of the chicken differ only in the amount of neuraminic acid bound to each isozyme. Law (1967) proposed that perhaps an enzyme system exists which attaches neuraminic acid units to alkaline phosphatase during its synthesis is responsible for the occurrence of alkaline phosphatase isozymes; the presence or absence of a gene controlling the attachment of neuraminic acid units could account for the observed dominance effects.

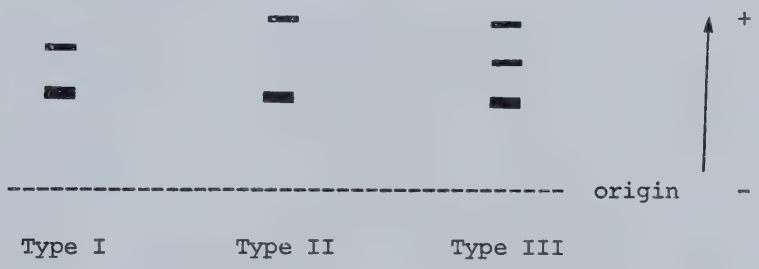
If Law's proposal is correct, then the occurrence of a fast-migrating and slow-migrating alkaline phosphatase isozymes is adequately explained. Unfortunately, my findings seem to suggest that this enzyme has more than two isozymic forms. My discovery of a double-banded phenotype (isozyme)implies that the mode of inheritance of alkaline phosphatase might not be as simple as that proposed by Law and Munro (1965). My findings seem to be in closer agreement with those of Tamaki and Tanabe (1970), but their observed double-banded variant may not be identical to mine. Only further experiments and breeding studies can resolve this uncertainty.

Chapter 3

Hemoglobin

Introduction

In 1968, Washburn reported the presence of a mutant hemoglobin in the Athens-Canadian randombred population of chickens which migrates electrophoretically faster than the normal hemoglobin. Using cellulose acetate membrane electrophoresis, the three hemoglobin phenotypes observed by Washburn may be shown diagramatically as follows:



Inheritance studies involving F_1, F_2 and backcross progeny revealed that differences in the minor hemoglobin components are due to a set of allelic codominant genes; individuals homozygous for either allele have a single minor component either fast or slow, in addition to the normal major hemoglobin component (Washburn, K. W., 1968).

In the present study, confirmation of the three hemoglobin phenotypes and the mode of inheritance of the marker as described by Washburn (1968) was sought.

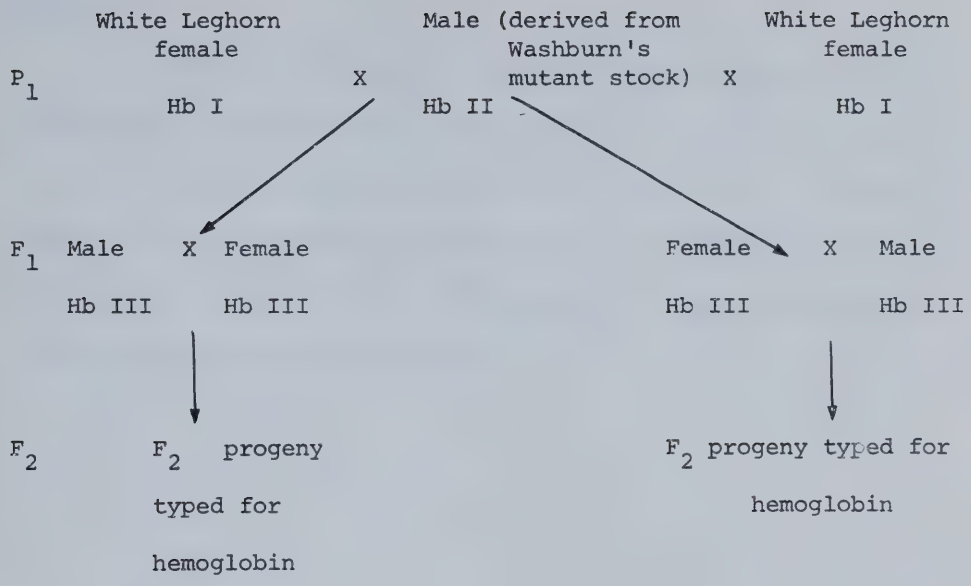
Materials and Methods

Hemoglobin samples from 19 White Leghorns of (both sexes) known B genotypes were subjected to cellulose acetate membrane electrophoresis according to the techniques described by Washburn (1968). The electrophoretic conditions used were:

1. Tris-EDTA-borate buffer, pH 9.6
2. 500 volts pulsed D.C., 300 pulses/second, 0.5 mfd., and 10 milliamperes.
3. Duration of the electrophoretic run = 45 min.

Following completion of the electrophoretic run, the cellulose acetate membranes were (for 15 minutes) stained in a solution consisting of 0.5g of Ponceau S, 7.5g of trichloroacetic acid, 7.5g of sulfosalicylic acid in 250 ml of distilled water. After staining, the cellulose acetate membranes were destained in 7 percent acetic acid.

Genetic confirmation of Washburn's findings (1968) was accomplished as follows. Two White Leghorn females possessing hemoglobin phenotype I were mated to a male of hemoglobin phenotype II (originally derived from Washburn's mutant stock). The resulting four F_1 progeny were tested for hemoglobin phenotype using the electrophoretic techniques described earlier. Next, the four F_1 progeny were set up as two mating pairs in separate breeding pens. All F_2 progeny resulting from these two mating pairs were typed for hemoglobin. The basic breeding scheme may be summarized as follows:


White Leghorn female
Male (derived from Washburn's mutant stock)
White Leghorn female
P1
X
Hb I
Hb II
X
Hb I
F1 Male X Female
Hb III Hb III
Female X Male
Hb III Hb III
F2 F2 progeny typed for hemoglobin
F2 progeny typed for hemoglobin

Results

All the 19 White Leghorns tested possessed hemoglobin Type I. No associations between hemoglobin type and B genotype are apparent.

The results of the breeding study are as follows. All the F_1 progeny tested possessed hemoglobin type III. In accordance with expectations, all three hemoglobin phenotypes were recovered from the F_2 progeny (Table 4 and Figure 17).

Discussion

Similar to Washburn's findings (1968), all the White Leghorns tested were found to have hemoglobin type I. Type I is the hemoglobin found in the majority of domestic fowl (Washburn, K. W., 1968), and hence it should be considered as the normal phenotype for the species.

The results of the breeding study demonstrated that all three phenotypic classes, namely type I, II, and III could be recovered in the F_2 generation (Table 4). The recovery of these three classes supports Washburn's claim (1968) that the observed hemoglobin types are controlled by a pair of co-dominant allelic genes.

All three hemoglobin types described by Washburn (1968) were observed (Figure 17). A major hemoglobin band was found in all three hemoglobin types. Type I and II possessed a single minor hemoglobin band of differing electrophoretic mobilities. Two minor bands were present in type III, one with the same migration rate as the minor hemoglobin band of type I and the other with same migration rate as the minor band of hemoglobin type II. The differences in electrophoretic mobilities of the minor hemoglobin bands are presumed to be due to differences in one or more amino acids in one of the globin polypeptides (Washburn, K. W., 1968).

Table 1. Results of Agglutination Tests of Erythrocytes From Chickens of Known B Genotypes.

Test Samples	No. Showing Agglutination Reaction	
	+	-
Males	0	26
Females	6	26

Table 1a. Results of Agglutination Tests of Erythrocytes from Female Chickens of Known B Genotypes.

B allele Represented in the Cells Tested	No. Showing Agglutination	
	+	-
$\underline{B}^{1}\underline{-}$	1	2
$\underline{B}^{2}\underline{-}$	2	10
$\underline{B}^{13}\underline{-}$	0	8
$\underline{B}^{14}\underline{-}$	1	2
$\underline{B}^{15}\underline{-}$	2	14
$\underline{B}^{19}\underline{-}$	1	6
$\underline{B}^{21}\underline{-}$	1	12

Table 2. Cell Electrophoretic Mobility Measurements of "Hi" Agglutinogen Positive and Negative Erythrocytes.

Trial NO.	Electrophoretic Mobility (u/sec/v/cm)			
	"Hi"$^{+}$ #1	"Hi"$^{+}$ #2	"Hi"$^{-}$ #3	"Hi"$^{-}$ #4
1.	1.46	2.16	0.76	1.35
2.	1.08	0.45	0.93	0.90
3.	0.64	1.17	1.00	0.91
4.	1.08	1.08	1.20	0.68
5.	0.74	0.86	1.15	1.06
6.	0.87	1.15	1.10	0.75
7.	0.72	1.35	0.96	0.76
8.	0.81	0.49	1.08	0.53
9.	0.74	1.04	0.90	0.86
10.	0.95	0.57	0.96	0.89
11.	0.74	1.04	0.77	0.80
12.	1.00	0.67	1.09	0.92
13.	1.06	1.04	0.78	0.79
14.	1.04	1.06	0.98	0.93
15.	0.72	0.93	0.70	0.63
16.	0.89	0.68	1.59	1.29
17.	0.70	0.90	0.76	0.64
18.	0.92	1.59	0.90	1.00
19.	0.74	0.95	0.68	0.61
20.	0.95	1.59	0.96	1.15
21.	0.86	1.35	0.68	0.73
22.	1.04	1.15	1.20	1.02
23.	0.68	0.55	0.90	0.77
Sample Size (n)	23	23	23	23
Mean	0.89	1.04	0.96	0.83
Standard Error	0.04	0.08	0.04	0.06

"Hi"$^{+}$ #1 = #14546 female "Hi"$^{+}$ #2 = #14428 female

"Hi"$^{-}$ #3 = #1333 male "Hi"$^{-}$ #3 = #13277 male

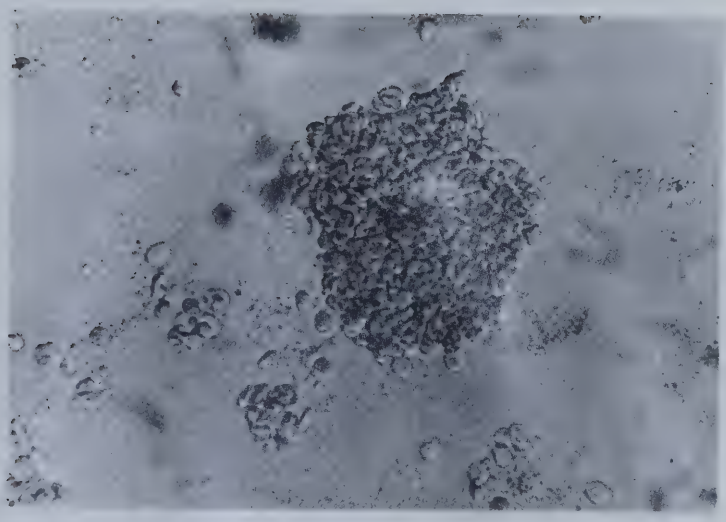
Table 3. The Observed Alkaline Phosphatase Phenotypes of Chickens of Known B Genotype

B Genotype	No. of Observed Phenotypes		
	Fast (F)	Slow (S)	Double (D)
B^{21}/B^{21}	3	--	--
B^{15}/B^{15}	1	4	--
B^{13}/B^{13}	1	--	1
B^{2}/B^{2}	--	3	1
B^{1}/B^{1}	--	3	2
B^{14}/B^{14}	--	4	--
B^{19}/B^{21}	--	--	2

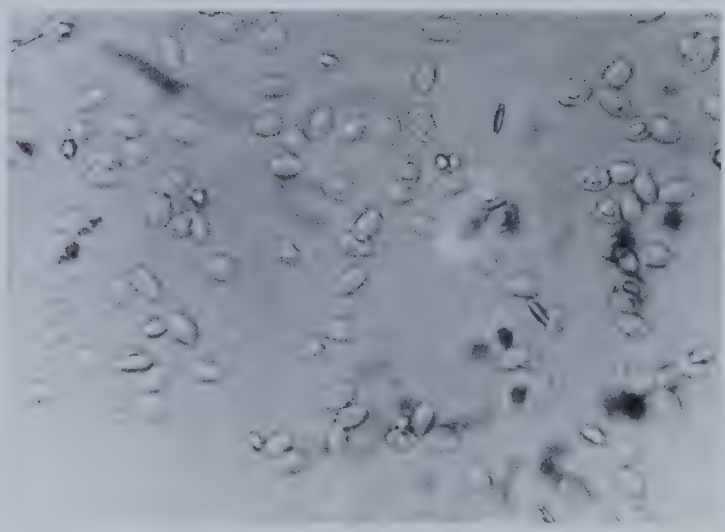
Table 4. Numbers of Progeny With Different Hemoglobin Phenotypes

Cross	Hemoglobin Phenotypes		
	Type I	Type II	Type III
P_1	1	1	0
F_1	0	0	4
F_2	5	5	8

Figure 1. Photomicrographs of typical agglutination of erytrhocytes induced by the agglutinins of the crude pea extract. Incubation of 0.5 ml of "Hi" agglutinogen-bearing cells (3.0×10^7 cells/ml) with 0.5 ml of pea extract (25 mg/ml in saline) for 1 hour at 37 C resulted in a typical agglutination reaction as shown in photograph (a). No agglutination of the same cells was observed when 0.5 ml of the cell suspension was incubated with 0.5 ml of saline for 1 hour at 37 as shown in photograph (b). Magnification 250X.

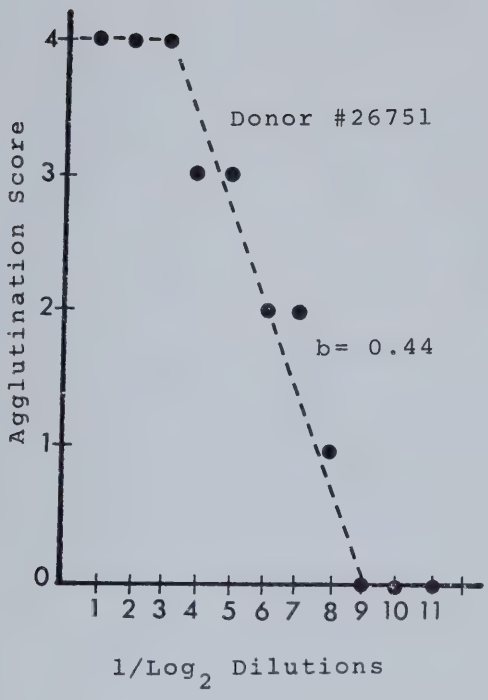


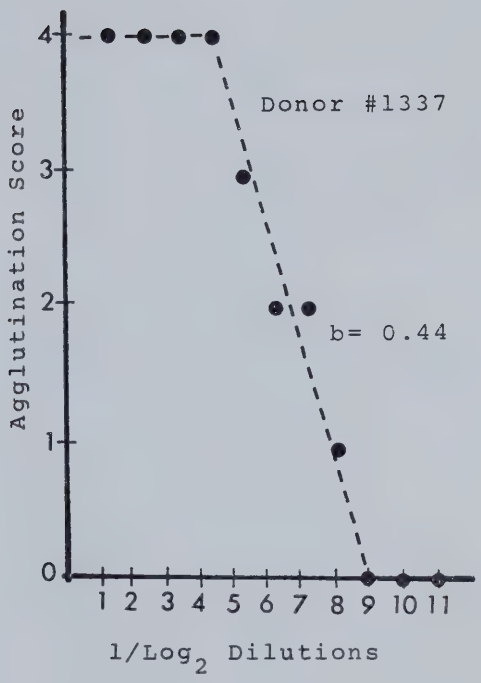
(a)

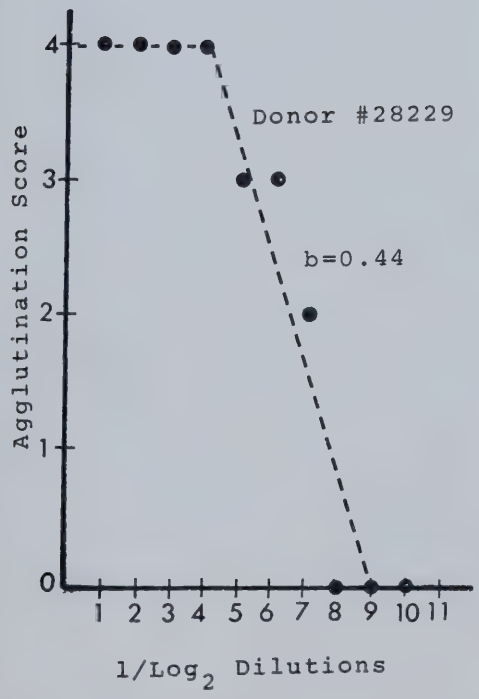


(b)

Figure 2. The effects of serial dilutions of the crude pea extract on the agglutination process. Half milliliter samples of "Hi" agglutinogen-bearing erythrocytes obtained from donors #26751, #1337, #28229, and #2427 were incubated with 0.5 ml of two-fold serial dilutions of pea extract for 1 hour at 37 C and agglutinations were assessed.


Donor #26751
b= 0.44
Agglutination Score
0
1
2
3
4
1 2 3 4 5 6 7 8 9 10 11
1/Log2 Dilutions


Donor #1337
b= 0.44
Agglutination Score
0
1
2
3
4
1 2 3 4 5 6 7 8 9 10 11
1/Log2 Dilutions


Donor #28229
b=0.44
Agglutination Score
0
1
2
3
4
1 2 3 4 5 6 7 8 9 10 11
1/Log2 Dilutions

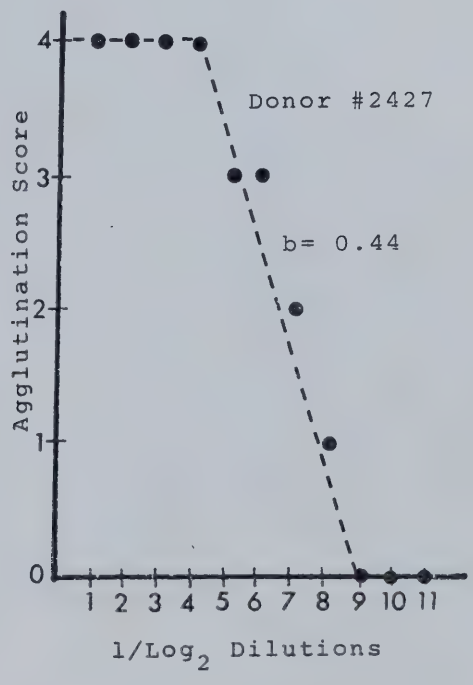

Donor #2427
b= 0.44
Agglutination Score
0
1
2
3
4
1 2 3 4 5 6 7 8 9 10 11
1/Log2 Dilutions

Figure 3. The inhibitory effects of D-mannose on the agglutination process induced by the agglutinins of the pea extract. Serial dilutions of 0.2M solutions of D-mannose and D-fructose were prepared with saline and the resultant sugar dilutions were each incubated with 0.5 ml pea extract for 1 hour at 37. Following incubation, 0.5 ml of "Hi" agglutinogen positive erythrocytes obtained from donor #1337 (3.0×10^7 cells/ml in saline) was added to each sugar dilution and agglutination was determined. The sugar dilutions are expressed in mg/ml (final concentration).

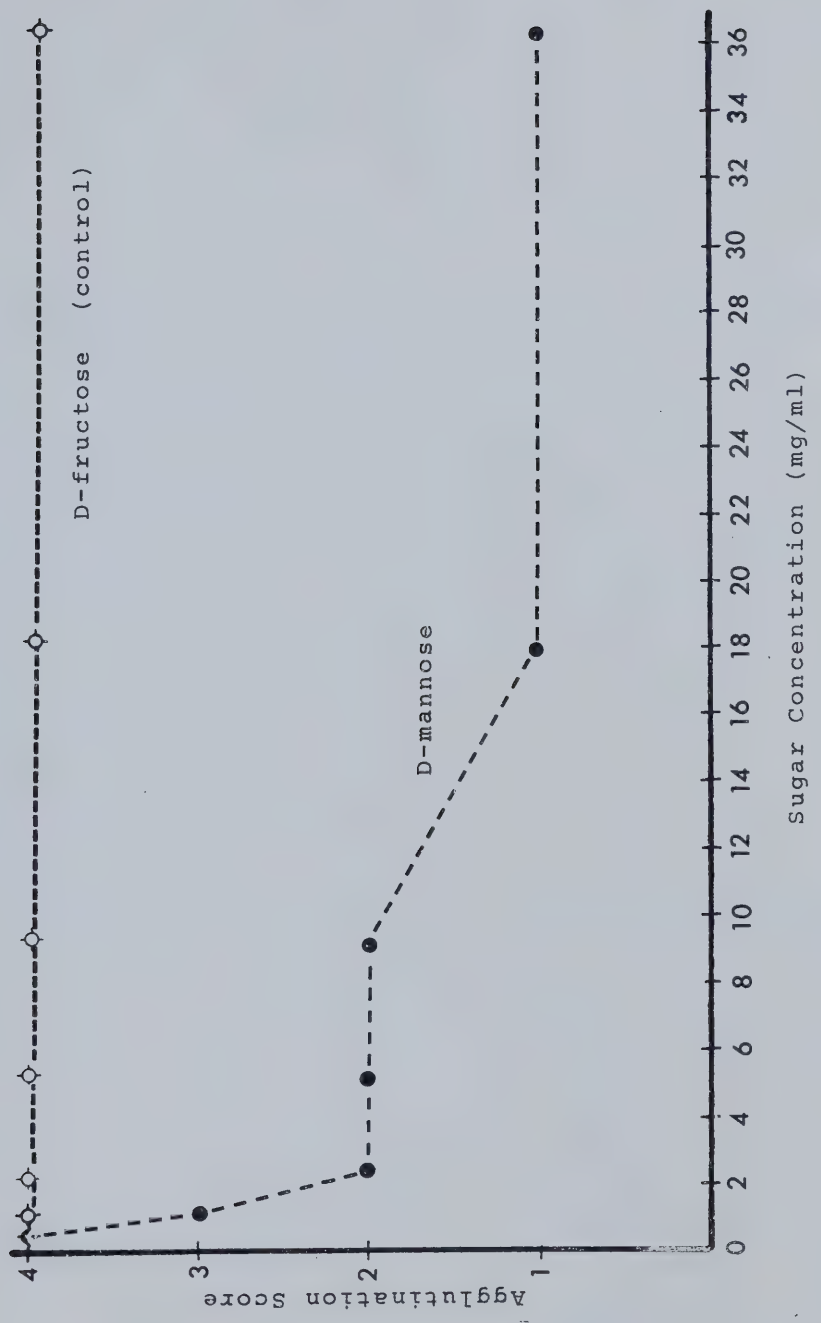

Agglutination Score
4
3
2
1
D-fructose (control)
D-mannose
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Sugar Concentration (mg/ml)

Figure 4. The effects of temperature on the agglutinating activity of the pea extract. Five 0.5 ml volumes of the crude pea extract were incubated at 37 C, 50 C, 56 C, and 90 C for a duration of 1 hour. Following the pre-incubation period, 0.5 ml of "Hi" agglutinogen positive erythrocytes from donor #14546 was added to each of the heat-treated pea extract and agglutination was assessed.

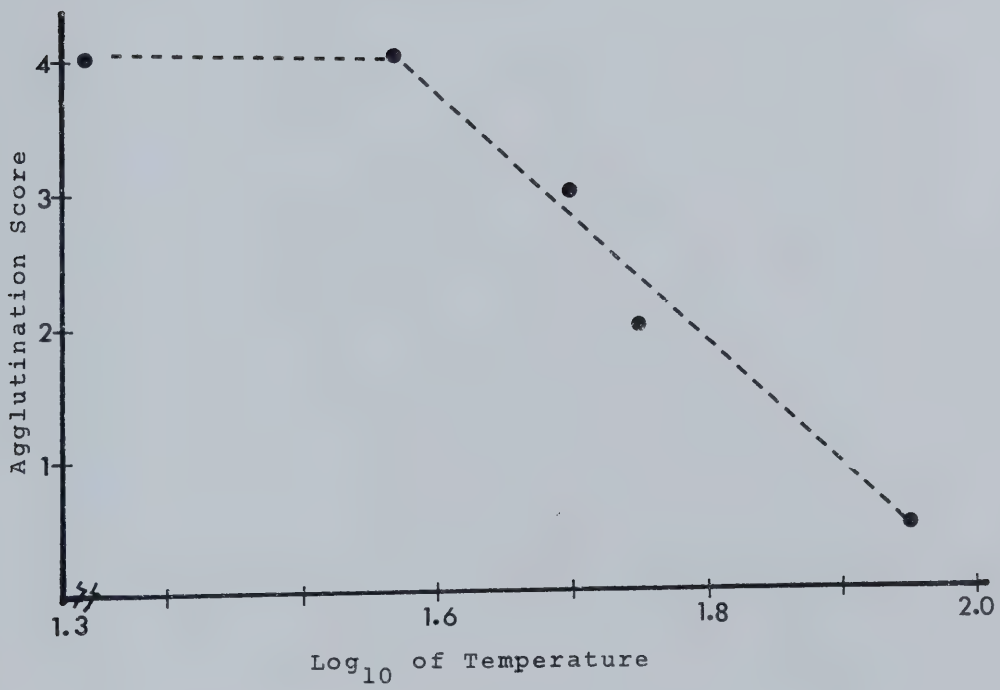

Agglutination Score
4
3
2
1
1.3
1.6
1.8
2.0
Log10 of Temperature

Figure 5. The effects of neuraminidase on the agglutination process induced by the agglutinins of the pea extract. Half milliliter dilutions of neuraminidase were incubated with 0.5 ml volumes of "Hi" agglutinogen positive erythrocytes (3.3×10^7 cells/ml in phosphate-buffered saline, pH 5.7) for 1 hour at 37 C. Following neuraminidase treatment, the cell mixtures were washed three times in saline and 0.5 ml of crude pea extract was added to each cell mixture and agglutination was assessed. Duplicate trials were carried out for each test dilution. The neuraminidase dilutions are expressed in Units/ml (final concentration). A Unit of activity is defined as the amount of enzyme which will liberate 1.0 micro-Mole of N-acetyl neuraminic acid per minute at pH 5.0 at 37 C.

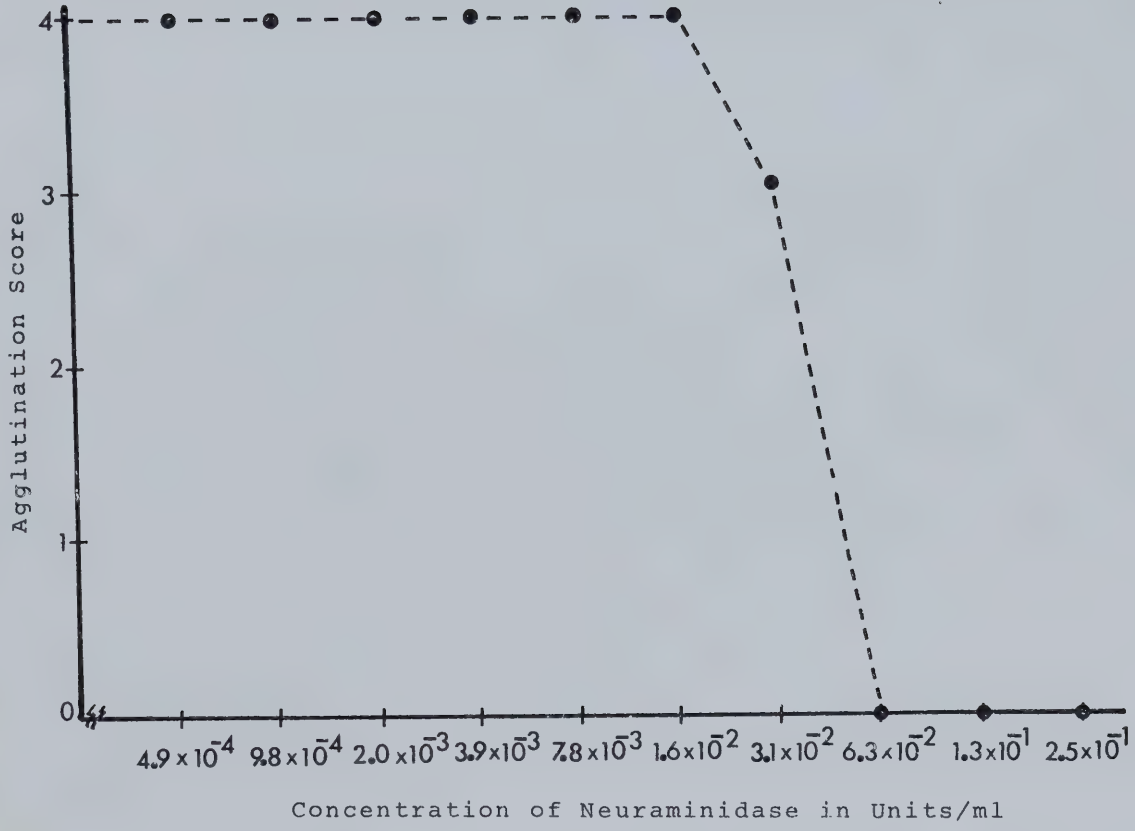

Agglutination Score
4
3
2
1
0
4.9 x 10⁻⁴
9.8 x 10⁻⁴
2.0 x 10⁻³
3.9 x 10⁻³
7.8 x 10⁻³
1.6 x 10⁻²
3.1 x 10⁻²
6.3 x 10⁻²
1.3 x 10⁻¹
2.5 x 10⁻¹
Concentration of Neuraminidase in Units/ml

Figure 6. The effects of pH on the agglutination process induced by agglutinins of the crude pea extract. Samples of 0.5 ml volumes of pea extract were added to 0.5 ml volumes of phosphate buffers of pH 5.7, 6.0, 6.5, 7.0, 7.5, and 8.0 and incubated for 1 hour at 37 C with 0.5 ml volumes of "Hi" agglutinogen positive erythrocytes from donor #4546. Following incubation, agglutination was assessed.

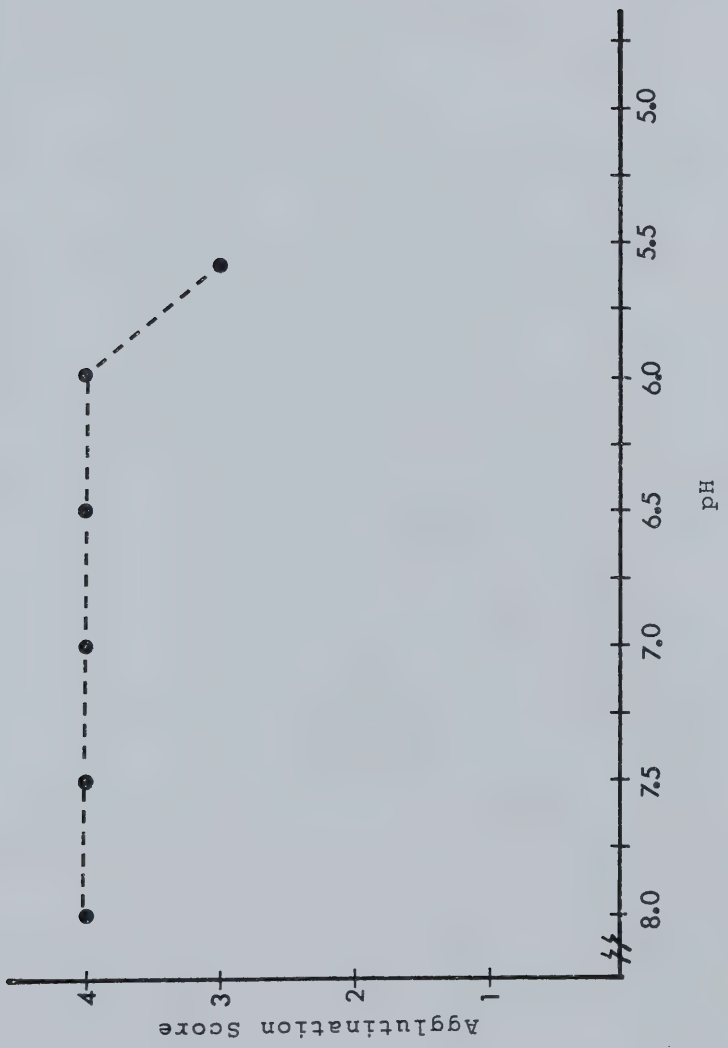

Agglutination Score
4
3
2
1
8.0
7.5
7.0
6.5
6.0
5.5
5.0
pH

Figure 7. Densitometric scan of a 20 microliter sample of crude pea extract (10 mg/ml in distilled water) which had been resolved by disc gel acrylamide electrophoresis and subsequently stained for proteins with 0.2% Buffalo Black (in 7% acetic acid). Electrophoresis was carried out on 7% acrylamide gels with Trisglycine buffer, pH 8.3, at room temperature and 300 volts pulsed D. C. for 1 hour with a current of 30 milliamperes. Scanning of the protein bands following staining was performed on a Model 52 - C Transmission Density Unit equipped with an automatic stage and a type C phototube connected to a Model 501-M Photometer (Photovolt Corp., 1115 Broadway, N. Y., N. Y.).

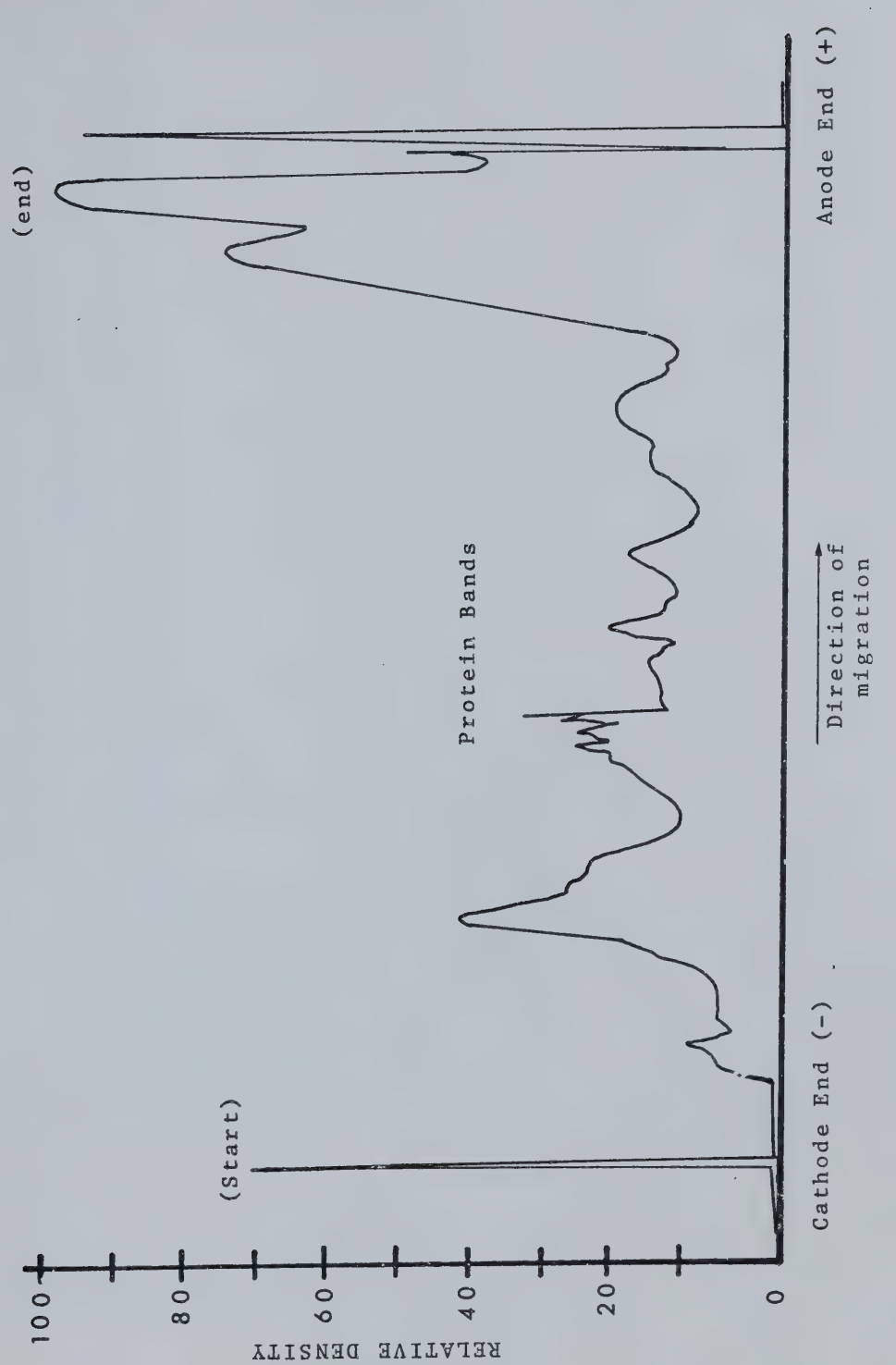

RELATIVE DENSITY
100
80
60
40
20
0
(Start)
(end)
Protein Bands
Cathode End (-)
Anode End (+)
Direction of migration

Figure 8. Elution profile of a sucrose density gradient centrifugation run. Samples of 5 mg of pea extract were layered on top of four 15-35% sucrose density gradients and high speed centrifugation was performed in a Beckman L2-65B Ultracentrifuge using a swinging bucket rotor (SW-27, Beckman Instruments Inc.). A speed of 25,000 RPM was maintained for 20 hours at a running temperature of 4 C. Following centrifugation, 20 drop fractions were collected from the bottom of each gradient. The fractions from all four gradients were pooled and the absorbance of the pooled fractions were measured at 280 nm. The major peak protein fractions were tested for agglutinating activity as described previously.

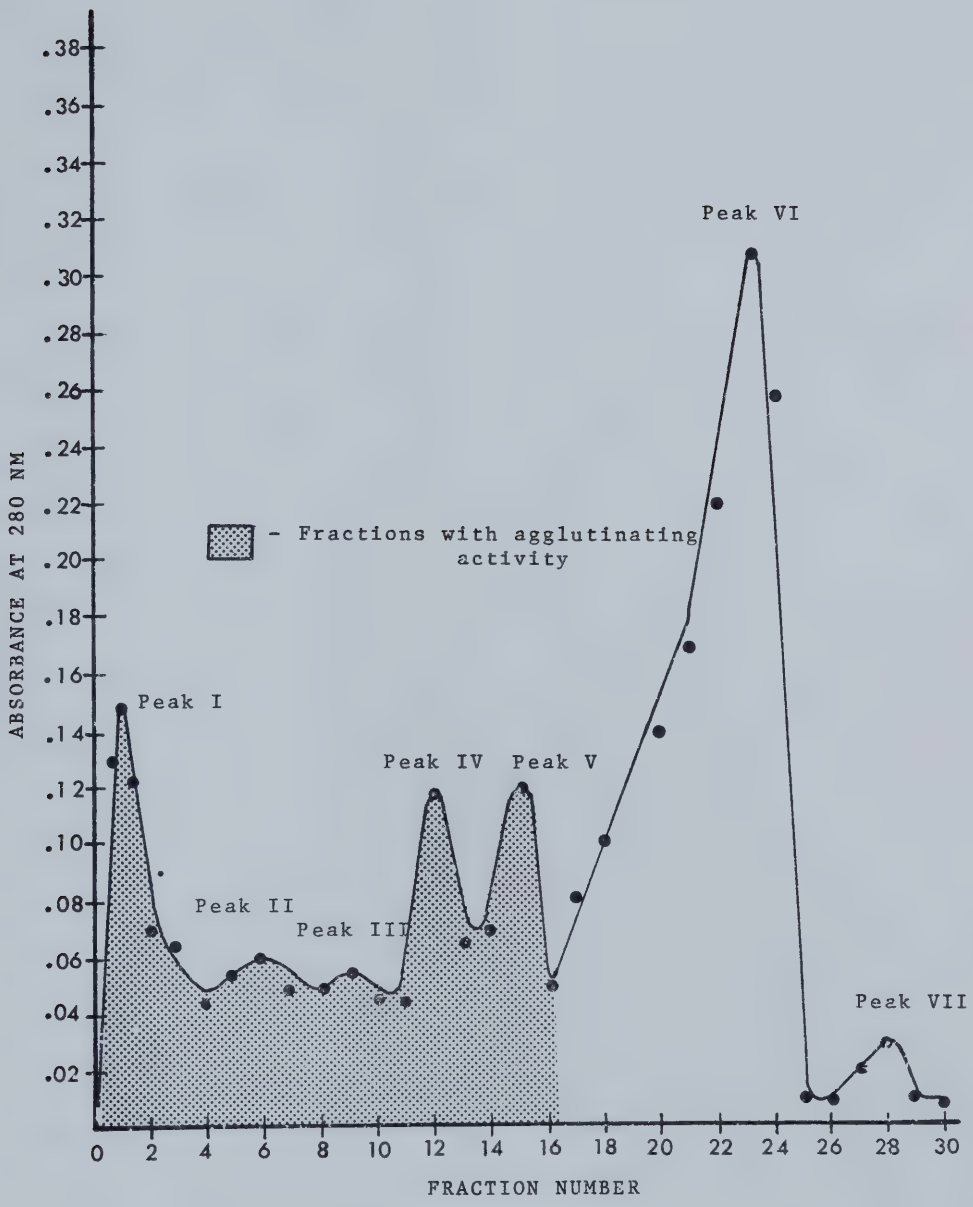

ABSORBANCE AT 280 NM
.38
.36
.34
.32
.30
.28
.26
.24
.22
.20
.18
.16
.14
.12
.10
.08
.06
.04
.02
- Fractions with agglutinating activity
Peak I
Peak II
Peak III
Peak IV
Peak V
Peak VI
Peak VII
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
FRACTION NUMBER

Figure 9. Elution profile of a 10 mg sample of crude pea extract subjected to Sephadex G-25 gel chromatographic analysis. A 10 mg sample of pea extract (dissolved in 2.0 ml of 0.85% NaCl) was applied onto a column (3 cm internal dia.X 45 cm) of Sephadex G-25. Elution was effected with 0.85% NaCl as an eluting buffer. Fifty drop fractions were collected and the absorbance of each fraction at 280 nm was measured on a spectrophotometer. The major protein fractions (Peaks) were tested for agglutinating activity as described previously.

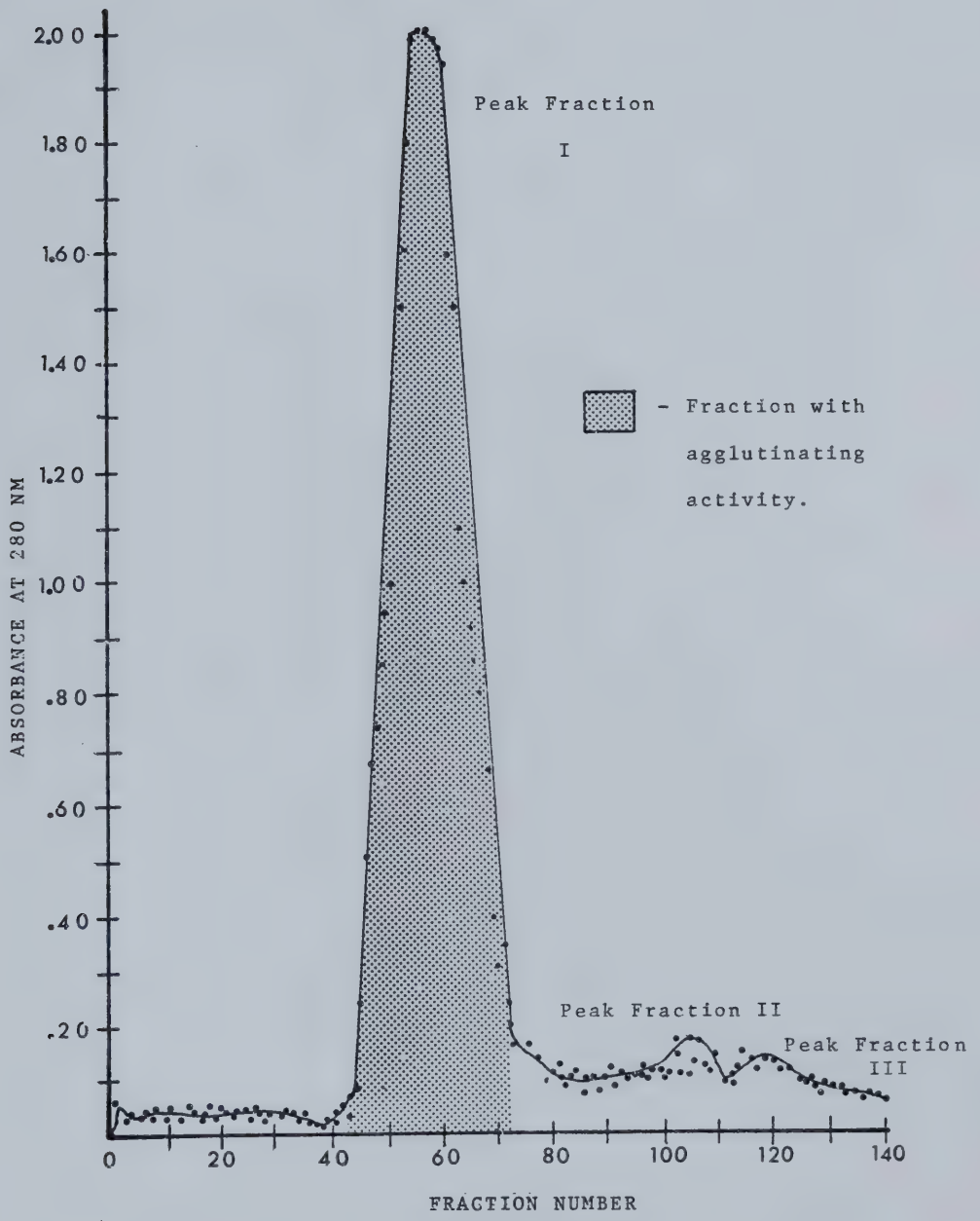

2.00
1.80
1.60
1.40
1.20
1.00
.80
.60
.40
.20
ABSORBANCE AT 280 NM
Peak Fraction I
- Fraction with agglutinating activity.
Peak Fraction II
Peak Fraction III
0
20
40
60
80
100
120
140
FRACTION NUMBER

Figure 10. Molecular weight estimates of agglutination-active Peaks IV and VI. The graph is a plot of relative mobilities versus $\log_{10}$ of molecular weights. Using the calibration curve derived from the protein standards, namely ferritin (horse spleen), aldolase, ribonuclease A, and chymotrypsinogen A, the molecular weights of Peaks IV and VI were estimated graphically as shown by the dotted lines. The estimates of two independent trials were as follows:

Trial #1

Peak IV = 6310 Daltons

Peak VI = 7080 Daltons

Trial #2

Peak IV = 7080 Daltons

Peak VI = 7944 Daltons

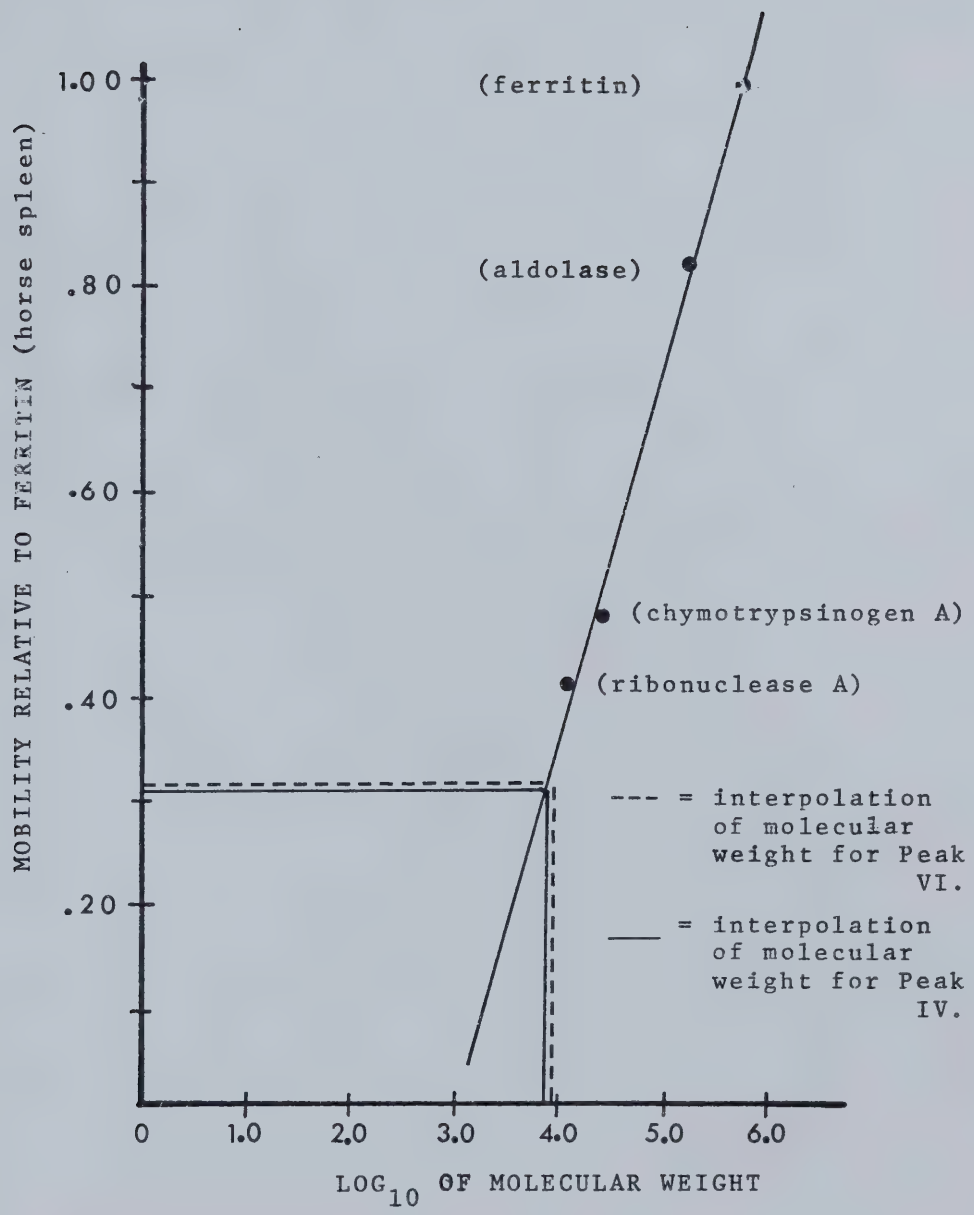

1.00
.80
.60
.40
.20
MOBILITY RELATIVE TO FERRITIN (horse spleen)
(ferritin)
(aldolase)
(chymotrypsinogen A)
(ribonuclease A)
--- = interpolation of molecular weight for Peak VI.
___ = interpolation of molecular weight for Peak IV.
0
1.0
2.0
3.0
4.0
5.0
6.0
LOG10 OF MOLECULAR WEIGHT

Figure 11. Migration positions of the agglutination-active components of Pisum arvense. Trial #1. This is a replica of the migration positions of Peaks IV and VI (agglutination-active components derived from the isoelectric focusing experiment) and protein standards obtained following SEPHADEX thin-layer gel filtration. The eluting buffer used was phosphate-buffered saline.

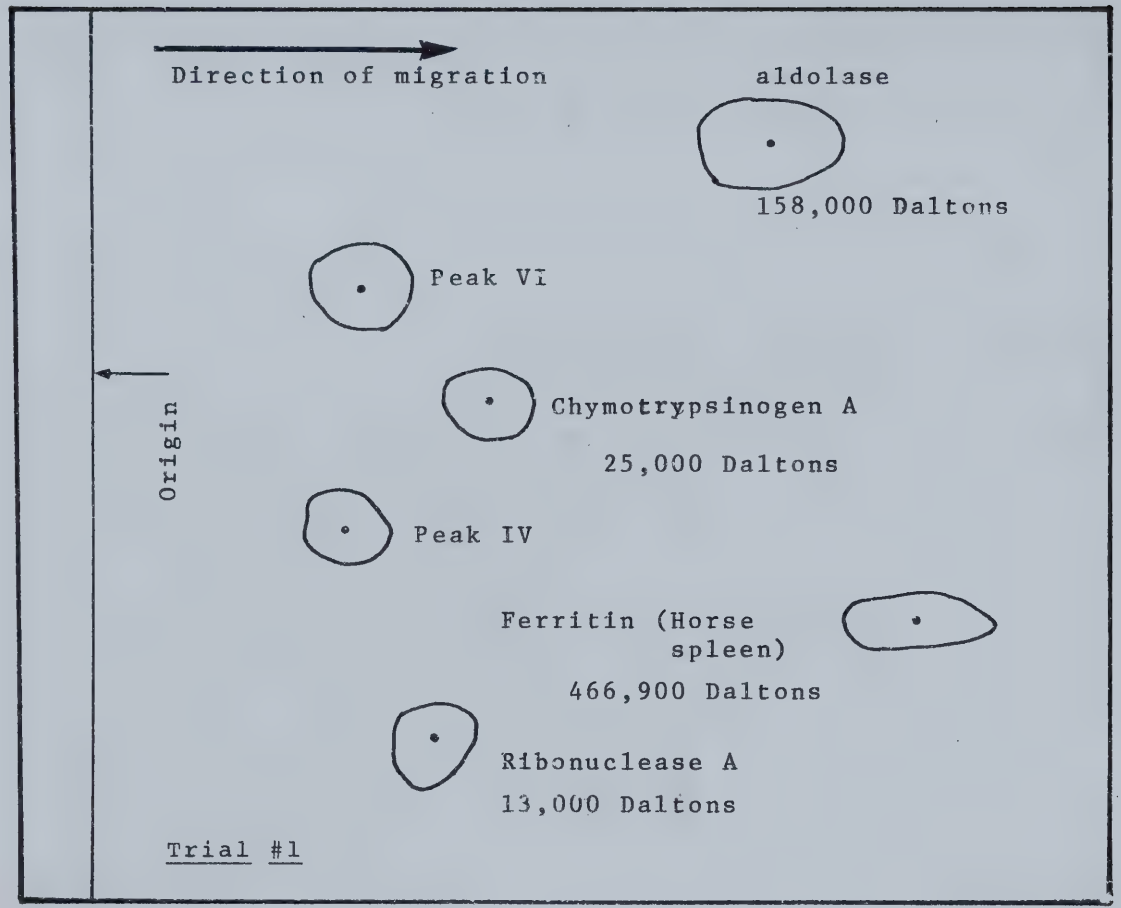

Direction of migration
aldolase
158,000 Daltons
Peak VI
Origin
Chymotrypsinogen A
25,000 Daltons
Peak IV
Ferritin (Horse spleen)
466,900 Daltons
Ribonuclease A
13,000 Daltons
Trial #1

Figure 12. Migration positions of the agglutination-active components of Pisum arvense. Trial #2. This is a replica of the migration positions of Peaks IV and VI (agglutination-active components derived from the isoelectric focusing experiment) and protein standards obtained following SEPHADEX thin-layer gel filtration. The eluting buffer used was phosphate-buffered saline.

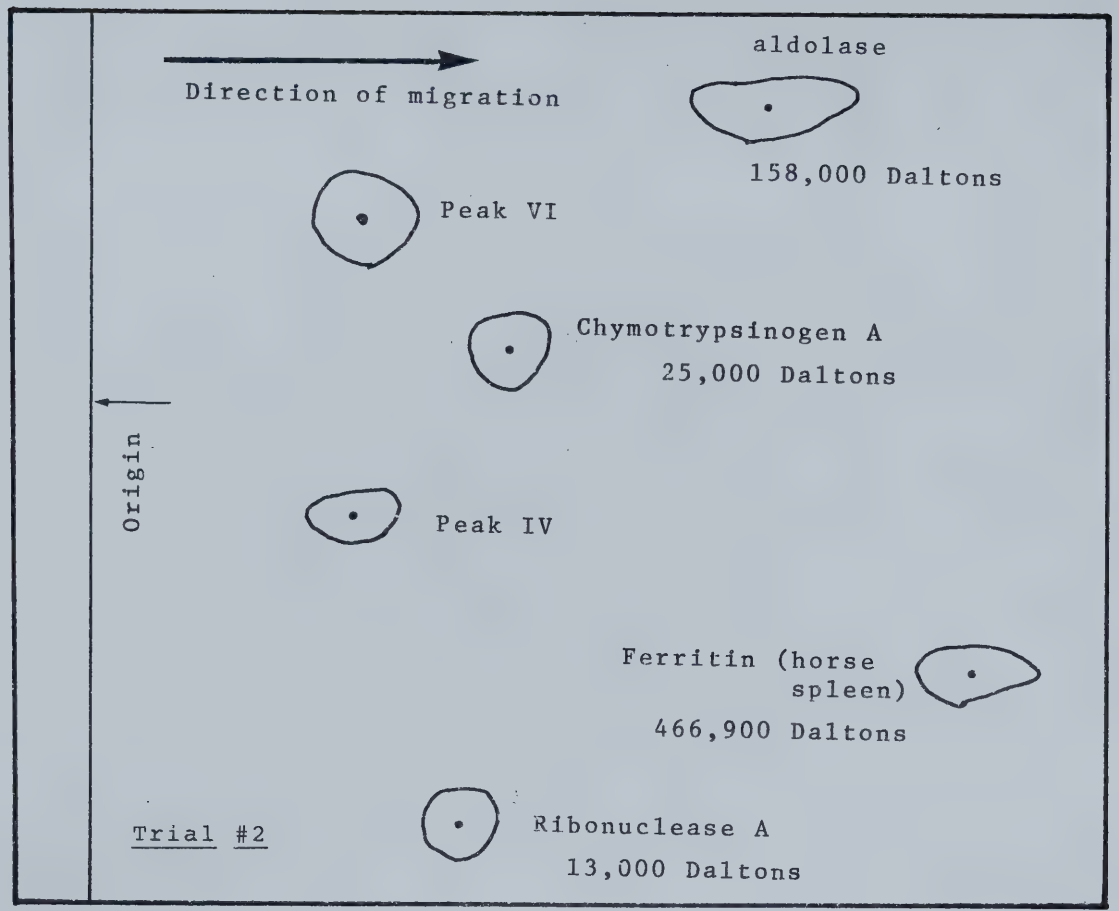

aldolase
Direction of migration
158,000 Daltons
Peak VI
Chymotrypsinogen A
25,000 Daltons
Origin
Peak IV
Ferritin (horse spleen)
466,900 Daltons
Trial #2
Ribonuclease A
13,000 Daltons

Figure 13. Photograph of protein bands observed in acrylamide gels following electrophoretic analysis of the major protein peak fractions (derived from the Sephadex gel chromatographic experiment) and samples of the crude pea extract. The electrophoretic conditions and staining procedures used were the same as those described earlier (See Materials and Methods section). Samples: a = Peak I b = Peak I c, d, e, and f = crude pea extract. Ten microliter volumes were applied for all samples.

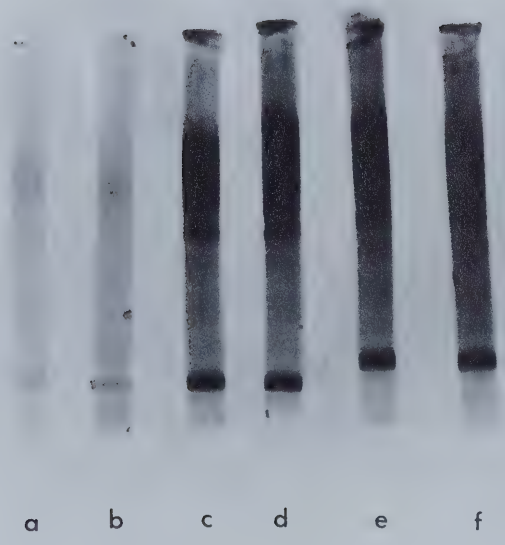

a
b
c
d
e
f

Figure 14. The results (elution profile) of an isoelectrofocusing analysis of the pea extract. A 10 mg sample of the crude pea extract in 1.0 ml of distilled water was applied to a sucrose gradient (440 ml) and subjected to isoelectrofocusing in a LKB 8100 isoelectrofocusing column (440ml) under the following conditions:

1. current - initial = 30 mA; final = 0.5 mA
2. voltage - initial = 300 v; final = 1200 v
3. power - initial = 9.0 Watts; final = 6.0 Watts
4. running temperature - 4 C
5. total duration of run - 24 hours.

Following the electrophoretic run, 70 fractions of 40 drops each (about 5.0ml) were collected and the absorbance of each fraction was measured at 280 nm. The pH's of the fractions were also measured and 0.5 ml samples of each major protein (Peaks) fractions were tested for agglutinating activity with 0.5 ml "Hi" agglutinogen positive erythrocytes from donor #14546.

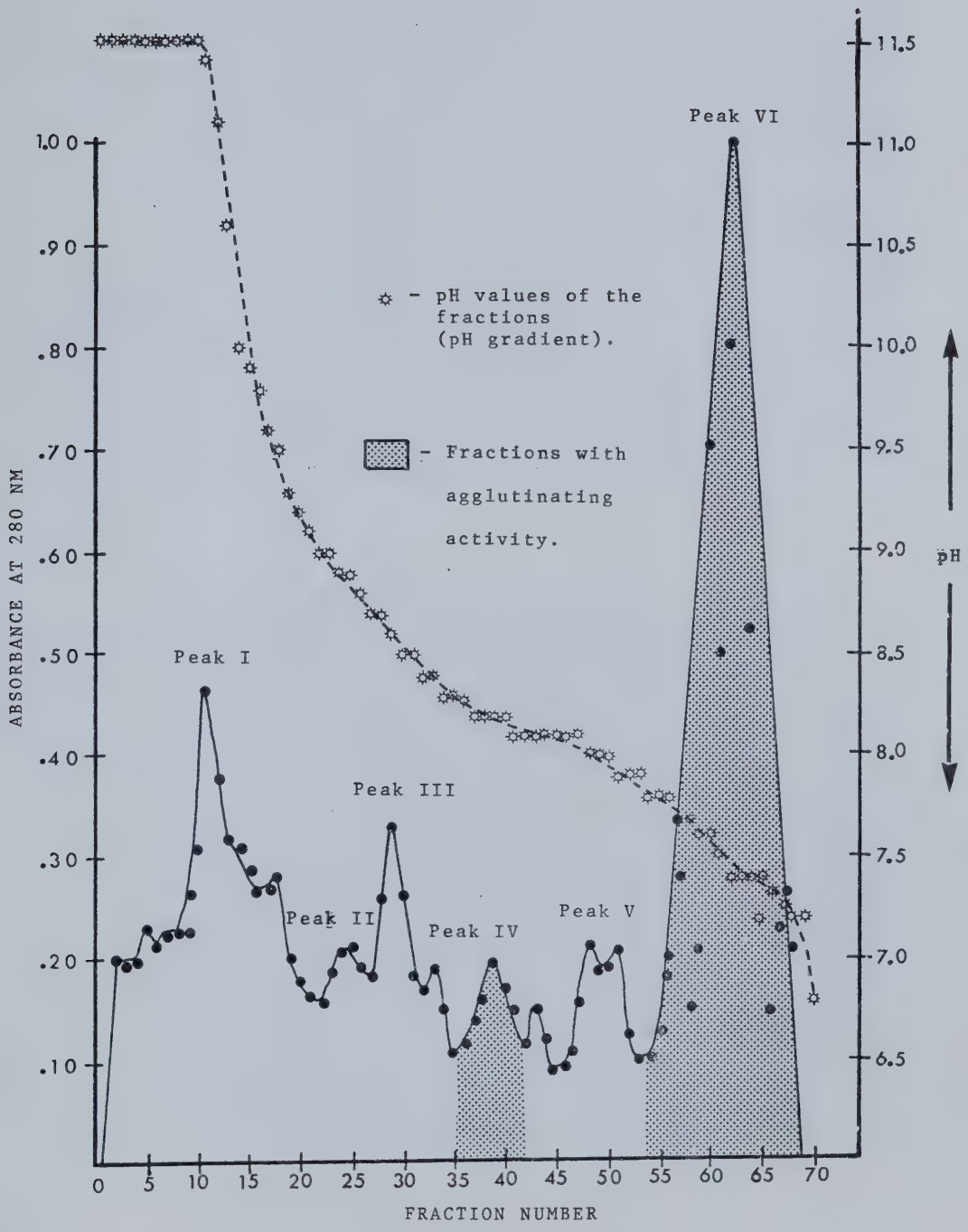
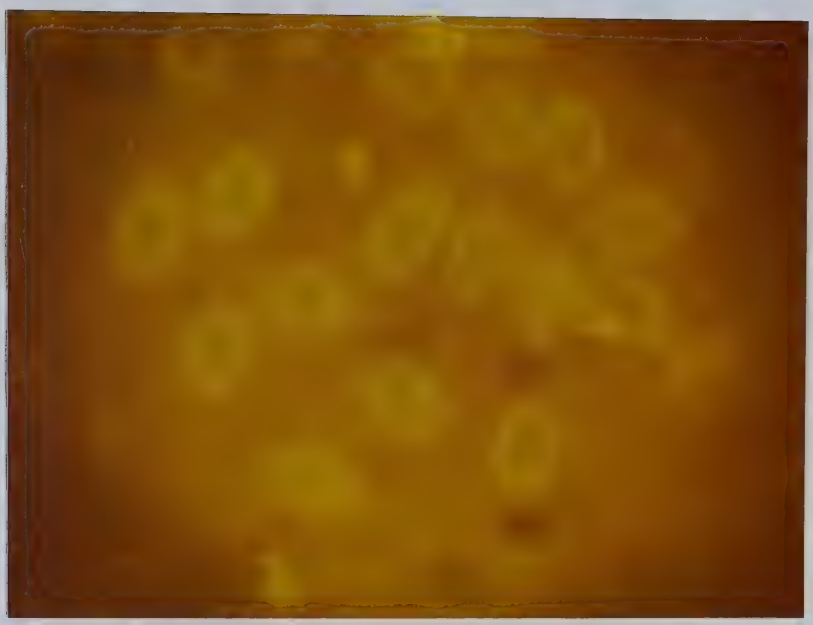

Peak VI
☼ - pH values of the fractions (pH gradient).
- Fractions with agglutinating activity.
Peak I
Peak III
Peak II
Peak IV
Peak V
ABSORBANCE AT 280 NM
1.00
.90
.80
.70
.60
.50
.40
.30
.20
.10
11.5
11.0
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
pH
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
FRACTION NUMBER

Figure 15. Photomicrographs of typical fluorescent "Hi" agglutinogen positive erythrocytes following incubation of these cells with fluorescein-labelled pea extract. Magnification = 1000X.



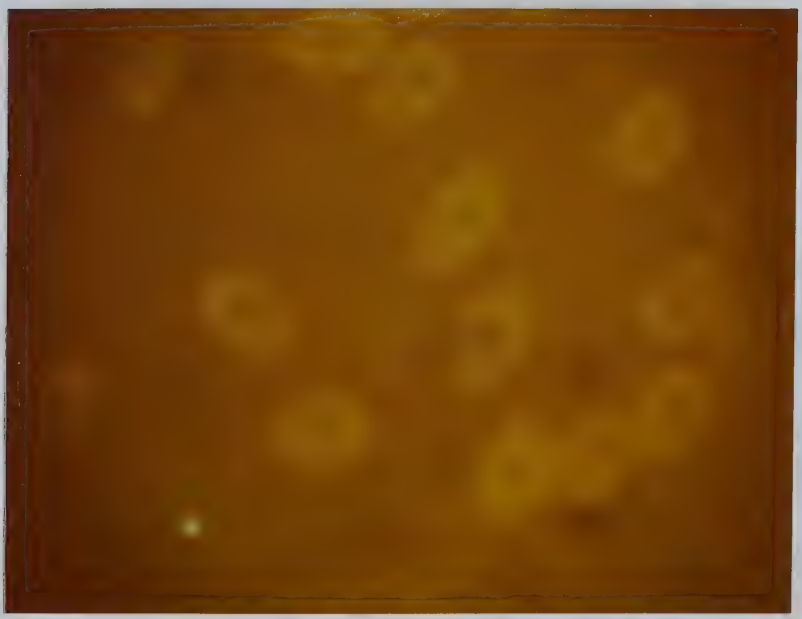


Figure 16. Alkaline phosphatase phenotypes. Disc gel electrophoresis was used to resolved the alkaline phosphatase phenotypes. The electrophoretic conditions used were:

1. Tris-borate buffer, pH 9.5
2. 300 volts pulsed D. C., 300 pulses per second
   1.0 mfd., 20 milliamperes
3. Duration of the electrophoretic run was 35 minutes.

The observed alkaline phosphatase phenotypes were:

S (slow-migrating) - e and f

F (fast-migrating) - c and d

D (double-banded) - a and b

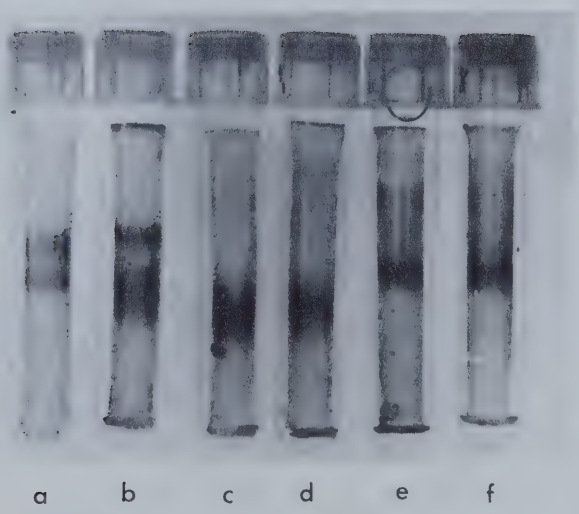

a
b
c
d
e
f

Figure 17. Hemoglobin types (phenotypes). Cellulose acetate membrane electrophoresis using Tris-EDTA-borate buffer, pH 9.6 was used to resolve the hemoglobin types. The electrophoretic conditions used were:

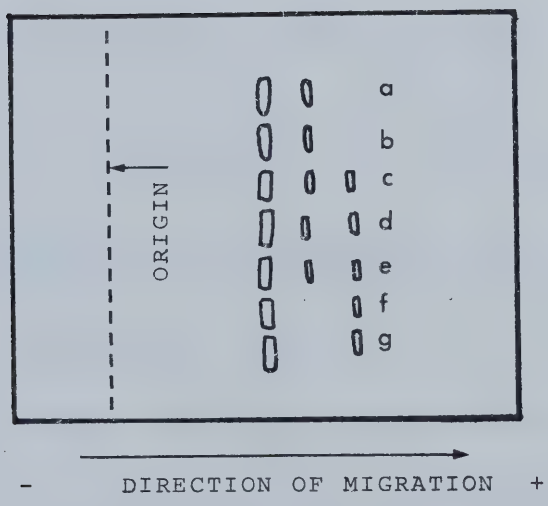
1. 500 volts pulsed D. C., 300 pulses per second, 0.5 mfd. and 10 milliamperes.
2. Duration of the electrophoretic run was 45 minutes.

The observed hemoglobin phenotypes were:

Type I = a and b

Type II = f and g

Type III = c, d, and e


ORIGIN
a
b
c
d
e
f
g
-
DIRECTION OF MIGRATION
+

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